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## A STUDY OF CERTAIN METALS IN THE PREVENTION OF NUTRITIONAL ANEMIA IN THE RAT

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(Received for publication, January 28, 1932)

The plan for investigating the effect of metals on nutritional anemia in the albino rat has, in general, been to study their possible curative action on the anemia produced by an unsupplemented milk diet. However, studies have been made in which a preventive procedure has been employed. Krauss (5), Waddell, Steenbock, and Hart (14), Elvehjem and Kemmerer (3) have, in the course of their experiments, used this method and have demonstrated that the addition of iron and copper to a whole milk diet serves to prevent a nutritional anemia in the rat, while Beard, Rafferty, and Myers (2) believe iron alone to be effective in prevention.

The preventive procedure offers the advantage that the effect of the metals is studied in well rather than in sick (anemic) animals, and that the action of the tested substance can be determined in a shorter experimental period. In our previous work (12) we began to use such a procedure by feeding purified iron from the beginning of the experiment and found that iron was ineffective in preventing the development of an anemia, confirming the observation of Krauss (5) and that of Titus and Hughes (11).

The purpose of this work was to study the preventive action of certain metals, including those which we had found to be ineffective in correcting nutritional anemia (12), and to determine whether the failure of these metals to cause an increase of hemoglobin in anemic animals might have been due to some toxic effect resulting from the comparatively high levels at which the metals were fed.

## EXPERIMENTAL

Extreme care was exercised throughout to avoid metallic contamination. Glass cages,<sup>1</sup> glass feeding dishes, and whole milk, from Holstein cows, collected *directly* into glass jars were used in this experiment. Each milking was personally supervised by one of us. Prior to assembling the cages, the glass parts were thoroughly washed, soaked in dilute nitric acid, rinsed with distilled water, with water redistilled from glass, and finally with alcohol redistilled from glass, and then allowed to drain until dry. The same process was always followed in washing the feeding pipettes and in preparing the glass jars for milk collection. The feeding dishes were treated in the same way except that the redistilled water rinse was omitted.

Male albino rats, 25 days old, were placed in individual glass cages and fed a diet of whole milk for 4 days. The metals to be tested were then fed as solutions of soluble salts, the same preparations previously used (12). These were added to a small amount of the milk in the morning 6 days a week, and, after this was consumed, more milk was given *ad libitum*. On Sundays the feeding of the metals was omitted, but the usual amount of milk was given. Care was taken to provide the animals with as much milk as they would drink. Weekly weight and hemoglobin determinations (Newcomer method) were made. Blood was obtained by clipping the tail and excessive bleeding was avoided by searing the tail on a hot glass plate.

Five groups of animals were studied. These groups and their respective supplements were as follows: Group I (seven rats), 0.5 mg. of iron; Group II (six rats), 0.5 mg. of iron and 0.025 mg. of copper; Group III (eight rats), 0.5 mg. of iron and 1.0 or 0.1 mg. of manganese; Group IV (seven rats), 0.5 mg. of iron with a mixture composed of 1.0 mg. of manganese, 0.5 mg. of cobalt, 1.0 mg. of nickel, and 0.5 mg. of zinc; and Group V (six rats), 0.5 mg. of iron, the same mixture of metals as Group IV, and 0.025 mg. of copper. With the exception of the 0.1 mg. level of manganese, the above amounts of supplementary metals are the lower levels fed in our previous work (12).

Since the rats of Groups IV and V showed an apparent distaste

<sup>1</sup> A description of the glass cage is given in an earlier paper (12).

for the amount of salts in their diet, some difficulty was encountered in securing quantitative consumption of the supplements during the first few days of the experiment. Thereafter, the iron and manganese (Group IV) and the iron, manganese, and copper (Group V) were fed in the morning, and when this was consumed, the remaining supplements were given in a small amount of milk in the afternoon, and finally a larger quantity of milk was fed at night. This procedure proved satisfactory in obtaining quantitative consumption.

### *Results*

From the results obtained on the animals receiving iron alone (Group I, Chart I) it is evident that the iron failed to prevent the development of an anemia. All of the animals presented low weight curves, the hemoglobin values decreased, and the rats became typically anemic, as do animals on an unsupplemented milk diet. These results confirm our previous observation (12) that iron alone is ineffective in preventing nutritional anemia, but in no way agree with the report of Beard, Rafferty, and Myers (2) that inorganic iron has a prophylactic action in this type of anemia.

In contrast to the ineffectiveness of iron alone, copper supplementing iron (Group II, Chart I) prevented the development of the anemia. The animals grew well and maintained an approximately normal hemoglobin level, showing that copper as a supplement to iron is just as effective in preventing as in correcting the anemia. These results agree with the recent findings of Waddell, Steenbock, and Hart (14).

When, in addition to iron, manganese was fed at a level of either 1.0 or 0.1 mg. (Group III, Chart II), no evidence that this metal exerted any hematopoietic effect was observed, thus demonstrating the inability of manganese to prevent nutritional anemia. It may be emphasized that the 0.1 mg. level of manganese, reported by Myers and Beard (9) to be the optimal dose of this element with 0.5 mg. of iron in the regeneration of hemoglobin in the anemic animal, is found without effect in preventing the anemia. There was observed, however, some beneficial effect on growth and on the length of life. In view of these observa-

## Prevention of Nutritional Anemia

tions, it is difficult to believe that either of these levels of manganese might have had any significant toxic action.

In all cases animals of the fourth group (Chart III) receiving

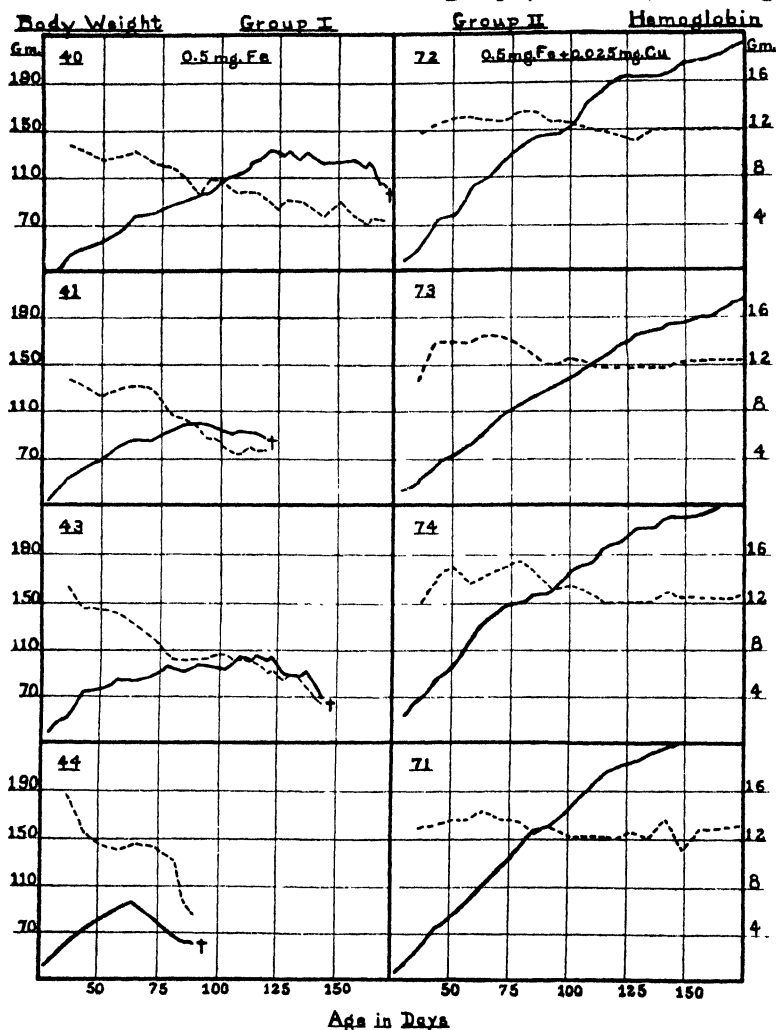


CHART I. Typical growth curves (solid lines) and hemoglobin curves (broken lines) of rats receiving whole milk plus iron (Group I), and of rats receiving whole milk plus iron and copper (Group II). The dagger indicates death.

the milk-iron diet supplemented by the mixture of manganese, cobalt, nickel, and zinc developed an anemia. The hemoglobin level progressively decreased until death, indicating that this mixture of metals is also unable to prevent the development of a nutritional anemia.

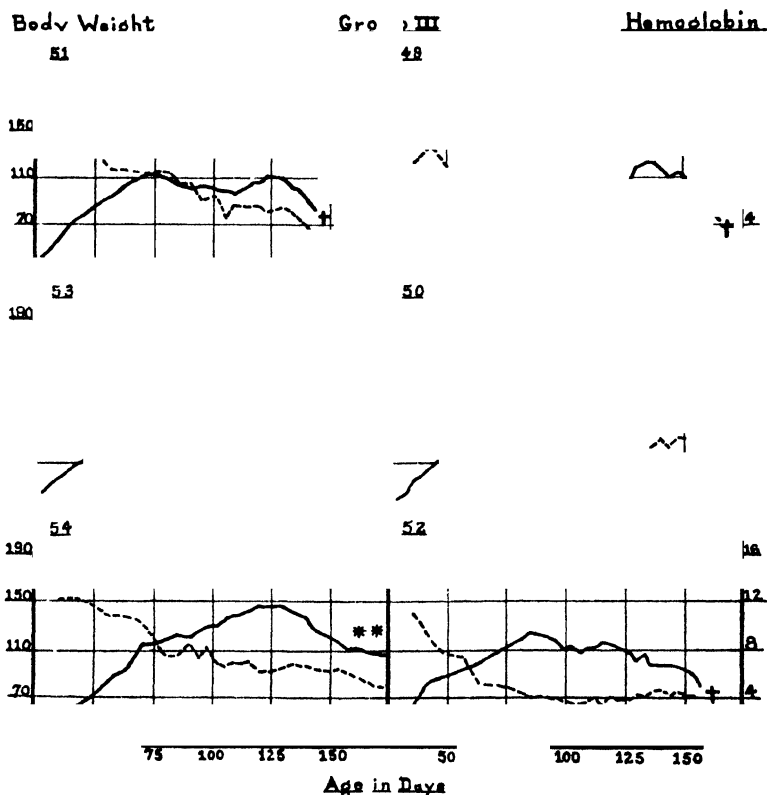


CHART II. Typical growth curves (solid lines) and hemoglobin curves (broken lines) of rats (Group III) receiving whole milk plus iron and manganese at a level of 1.0 or 0.1 mg. The dagger indicates death. The asterisk indicates death at the age of 196 days. The double asterisk indicates that the diet was changed on the 197th day.

When copper was given in addition to the manganese, cobalt, nickel, and zinc, in no case did the animals (Group V, Chart III) show a decrease in hemoglobin, but a normal level or one some-

what above normal was maintained until 3 to 5 weeks after the experiment was begun, when a definite increase above normal occurred. This phase of the problem is discussed in the following

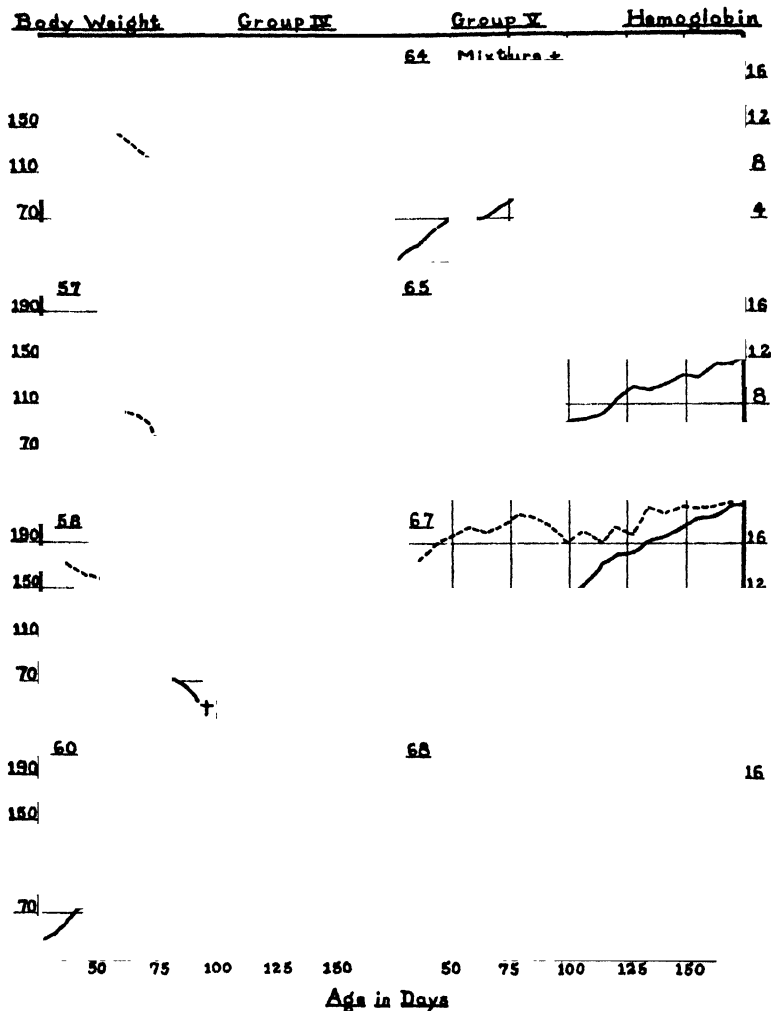


CHART III. Typical growth curves (solid lines) and hemoglobin curves (broken lines) of rats receiving whole milk plus iron and a mixture of manganese, cobalt, nickel, and zinc (Group IV), and of rats receiving whole milk plus iron, the same mixture of metals, and copper (Group V). The dagger indicates death.

paper (10). The weights of these animals were somewhat lower than those of the animals receiving only copper and iron (Group II, Chart I).

#### DISCUSSION

In our earlier experiments (12), manganese, cobalt, nickel, and zinc, respectively, when used as a supplement to iron, failed to cause a regeneration of hemoglobin in anemic animals. The data presented in the present paper show that this result cannot be explained on the assumption that a toxic action has obscured any favorable effect which the metal may have had on the hemoglobin level. A comparison of the hemoglobin curves of the rats receiving supplements of iron and copper only (Group II, Chart I) with those of the animals of Group V (Chart III), in which a mixture of manganese, cobalt, nickel, and zinc was fed in addition to the iron and copper, shows that the action of the copper was in no way impaired by the presence of this mixture of metals. A similar comparison of Groups IV and V (Chart III) warrants the conclusion that this mixture of metals, at the levels fed, did not possess sufficient toxic action to obscure any beneficial effect similar to that of copper that any one of these metals may have had. Thus, since the metals were given at the same levels in this and in the earlier series of experiments, it follows that their failure to cause a regeneration of hemoglobin in anemic animals could not have been due to any toxic action resulting from the comparatively high levels at which they were fed. Furthermore, the recent findings of Keil and Nelson (4), that cobalt, nickel, and zinc at 0.05 mg. levels and manganese at a 0.1 mg. level, each supplementing 0.5 mg. of iron, failed to stimulate hemoglobin regeneration in the anemic rat, support our belief that our failure to obtain an increase in hemoglobin with these same metals fed in greater amounts was due to the inertness of the metals rather than to a toxic effect.

It should be emphasized that all of our animals have been followed either until death or for a period of at least 4 months before conclusions have been drawn. During this time, in both the preventive and curative experiments, every animal that did not receive copper as a supplement to iron developed an anemia and died, while all animals receiving copper under conditions otherwise identical have lived and grown satisfactorily.



Our collective results in the study of nutritional anemia, obtained by employing a technique carefully designed so as to avoid traces of metallic contamination in all details of the work and by using sufficient numbers of animals to rule out individual variations, have invariably shown that iron alone and iron supplemented by certain metals other than copper are ineffective in either producing or maintaining an approximately normal hemoglobin level in rats on a whole milk diet, whereas copper supplementing iron is the only metal thus far found that has the ability to effect these results. These findings support the view held by Waddell, Steenbock, and Hart (13), Krauss (5, 6), Lewis, Weichselbaum, and McGhee (7), and Keil and Nelson (4) that copper is a necessary supplement to iron in the regeneration of hemoglobin in anemic rats. On the basis of our experimental results we must disagree with the contention of Beard and Myers (1) and Mitchell and Miller (8) that unsupplemented iron has an erythropoietic effect, and with the report of Beard, Rafferty, and Myers (2) that iron alone has both a prophylactic and curative action. In spite of this contradictory evidence, the invariable results obtained in our experiments convince us of the validity of the theory first proposed by the Wisconsin workers (13) that copper as a supplement to iron is essential to the regeneration of hemoglobin in rats made anemic by an exclusive milk diet.

#### SUMMARY

1. Inorganic iron fails to prevent the development of a nutritional anemia in the rat on a milk diet, whereas iron supplemented by copper permits the maintenance of an approximately normal hemoglobin level.

2. Iron supplemented by manganese fails to prevent the development of nutritional anemia.

3. A mixture of manganese, cobalt, nickel, and zinc, supplementing iron, has no prophylactic action in nutritional anemia, while the same mixture plus copper prevents a decrease in the hemoglobin level.

4. Evidence of any significant toxic effect of manganese, cobalt, nickel, or zinc was not observed.

5. Of all the metals studied, copper alone has the ability to supplement iron in preventing the nutritional anemia of the rat.

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## **POLYCYTHEMIA IN THE RAT ON A MILK-IRON-COPPER DIET SUPPLEMENTED BY COBALT**

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(Received for publication, January 28, 1932)

A study of the effect of certain metals on the prevention of nutritional anemia in the rat has been reported in the preceding paper (4). Animals receiving a mixture of manganese, cobalt, nickel, and zinc with iron developed a progressive anemia and died, whereas those receiving the same mixture with iron and copper developed an abnormally high hemoglobin value at the age of 7 to 9 weeks. After the high hemoglobin levels had persisted about 18 weeks, further blood studies (cell volume, erythrocyte, leucocyte, and differential leucocyte counts) were made on the group of rats receiving the mixture of metals with iron and copper, on a group receiving only iron and copper (Group II of the preceding paper (4)), and on a group of normal stock rats of comparable age. The results, which have been given in a preliminary report (3), definitely showed a polycythemia in all of the rats receiving the mixture of metals with iron and copper, while the rats receiving only iron and copper showed values approximately the same as those of the stock rats. It was evident, therefore, that one of the metals, manganese, cobalt, nickel, or zinc, or some combination of these metals, was acting with iron and copper to produce the polycythemia. The present study was made to determine which one or combination of these metals was responsible for this marked increase in hemoglobin and in red blood cells.

### **EXPERIMENTAL**

Thirty albino rats, 21 days old, were placed in glass cages<sup>1</sup> with their mothers, and fed whole milk only until the 25th day. The

<sup>1</sup> A description of the glass cage is given in an earlier paper by Underhill, Orten, and Lewis (5).

mothers were removed some time during the 4 day period. The young rats were then placed in individual glass cages and given a basal diet consisting of whole milk with 0.5 mg. of iron plus 0.025 mg. of copper daily until the 30th day, at which time the other supplementary metals, manganese, cobalt, nickel, and zinc,

TABLE I

*Blood Findings in Control Rats and Rats Fed a Milk-Iron-Copper Diet Supplemented by Various Other Metals\**

Group No.	Supplement to milk-Fe-Cu diet	Body weight	Hemo- globin	Cell volume	Erythrocytes per c.mm.
		<i>gm.</i>	<i>gm. per 100 cc.</i>	<i>per cent</i>	
1	None	242	11 8	47	7,500,000
2	Stock diet only	317	13 2	53	8,300,000
3	Mn	237	13 2	51	9,200,000
4	Co	155	18 2	74	14,400,000
5	Ni	209	12.4	50	8,500,000
6	Zn	224	11.0	46	8,200,000
7	Mn, Co	171	17.7	74	13,100,000
8	" Ni	198	13.6	56	9,300,000
9	" Zn	205	13.1	54	9,600,000
10	Co, Ni	163	17.2	71	12,100,000
11	" Zn	173	17 8	72	11,200,000
12	Ni, "	205	11 9	47	7,200,000
13	Mn, Co, Ni	187	18 9	70	12,000,000
14	" " Zn	197	18.1	70	11,300,000
15	Co, Ni, "	124	18.8	73	11,700,000
16	Mn, " "	214	13 8	56	7,700,000
17	" Co, Ni, Zn	180	19.5	79	11,800,000
Average for non-cobalt groups.....		216	12.5	51	8,300,000
Average for cobalt groups...		169	18 3	73	12,200,000

\* All figures except for body weights are averages of two determinations, the second of which was made when the rats were 136 to 139 days of age. Weights are averages of those taken on the 139th day.

were added. These were fed as solutions of soluble salts and at the following levels: manganese, 1.0 mg., as  $\text{MnSO}_4$ ; cobalt, 0.5 mg., as  $\text{CoCl}_2$ ; nickel, 1.0 mg., as  $\text{NiSO}_4$ ; zinc, 0.5 mg., as  $\text{ZnSO}_4$ . The salts were the same preparations as used in the work reported by Underhill, Orten, and Lewis (5). The metals were fed singly

and in all possible combinations to fifteen different groups as shown in detail in Table I. By this arrangement, although there were only two rats to a group, each metal was fed to sixteen animals. In addition, two groups of rats were maintained as controls, one (three rats) on the basal diet only, the other (seven rats) on a stock diet. Except in the case of the animals on a stock diet the same care and feeding technique as described in the foregoing paper (4) were used. Weights were determined weekly and blood studies were made biweekly. Blood was obtained by clipping the tail, and excessive bleeding was prevented by searing the tail on a hot glass plate.

Hemoglobin was determined by the improved Newcomer hemoglobinometer (Bausch and Lomb) with the blue filter. Hematocrit determinations were made with Van Allen pipettes and 1.3 per cent sodium oxalate solution as the diluting fluid. Erythrocyte counts were made with Thoma pipettes and a 1:200 dilution of blood with Hayem's solution. Leucocyte counts were made in a similar manner with a 1:20 dilution of blood in 2 per cent acetic acid. A Levy-Hausser hemocytometer chamber with the improved Neubauer ruling was used for the counting. Differential leucocyte counts were determined on thin blood smears prepared by the two slide method and stained with Wright's stain. 100 cells were counted diagonally lengthwise across the film.

The results of this study are summarized in Table I. The values given are group averages made from the last two determinations. The age of the animals at the time of the last determination was 136 to 139 days. From the data presented it is evident that the animals receiving the basal diet and *cobalt*, either alone or in combination with one or more of the other metals, exhibited a polycythemia, while all animals that did not receive cobalt maintained an approximately normal blood picture. The lower part of Table I gives composite averages for the cobalt and non-cobalt groups, respectively, and emphasizes the marked increase in the hemoglobin, erythrocyte, and cell volume values as a result of cobalt administration. These averages also show that the weights of those animals receiving cobalt were definitely lower than those of the non-cobalt groups. No significant variations were found in the leucocyte and differential leucocyte counts, and these determinations are, therefore, omitted in Table I.

In order to confirm the results obtained with  $\text{CoCl}_2$ , the action of  $\text{CoSO}_4$  was studied in a second series of rats with the same procedure and feeding technique. Group I-A (four rats) was given the basal diet of whole milk, 0.5 mg. of iron, and 0.025 mg. of copper daily; Group II-A (five rats), the same diet supplemented by 0.5 mg. of cobalt as  $\text{CoSO}_4$ . In Table II are given the group averages obtained from the most recent determinations made on these animals at the age of 136 to 139 days. Abnormally high hemoglobin, erythrocyte, and cell volume values with accompanying low weights were observed in all of the animals receiving the  $\text{CoSO}_4$ , while no blood values above normal were found in any of the control animals (Group I-A). No significant alterations

TABLE II  
*Blood Findings in Rats Fed Milk-Iron-Copper Diet Supplemented by Cobalt Sulfate\**

Group No.	Supplement to milk-Fe-Cu diet	Body weight	Hemoglobin	Cell volume	Erythrocytes per c mm.
		gm.	gm. per 100 cc	per cent	
I-A	None	236	12.2	52	7,900,000
II-A	$\text{CoSO}_4$	180	17.0	73	10,700,000

\* Averages of determinations made when the rats were 136 to 139 days of age.

occurred in the leucocyte and differential leucocyte counts. The results with  $\text{CoSO}_4$  are identical with those obtained with  $\text{CoCl}_2$ .

All animals receiving cobalt, either as  $\text{CoCl}_2$  or  $\text{CoSO}_4$ , presented a striking appearance. The ears, eyes, and paws became a deep red color in contrast to the pink color of the rats of the non-cobalt and normal groups. The difference in appearance was so marked that the cobalt-fed animals could be distinguished easily at sight. Blood from the tail had a very deep red color, was extremely thick, and clotted rapidly. As long as cobalt has been administered, the polycythemia has persisted. Two animals have been kept polycythemic by cobalt feeding (0.5 mg. of cobalt as  $\text{CoCl}_2$  daily) for a period of 330 days.

There has been only one fatal case, Rat 67 (Chart III of the preceding paper (4)). This animal ate and grew exceptionally

well until about 2 weeks before death which occurred at 200 days of age. A few days before death the animal showed a hemoglobin value of 25 gm., an erythrocyte count of 17,050,000, and a cell volume of 87 per cent. Autopsy revealed no gross abnormalities other than marked general engorgement of blood vessels and atrophy of the testes.

Preliminary studies on the effect of removing cobalt from the diet of animals kept polycythemic for approximately 220 days have shown that the hemoglobin, erythrocyte, and cell volume values return slowly toward normal.

#### DISCUSSION

During a study of the toxicity of various metals, Waltner and Waltner (6) found that a polycythemia was produced either when pulverized metallic cobalt in amounts of 0.5 per cent or 2 per cent was added to the mixed diet of normal rats, or when doses of 0.01 or 0.1 gm. of  $\text{CoCl}_2$  or  $\text{Co}(\text{NO}_3)_2$  were injected subcutaneously. An increase in hemoglobin and in erythrocytes as a result of the oral administration of cobalt to dogs was also observed by Mascherpa (1), who attributed the blood changes to a stimulation of the bone marrow. Myers, Beard, and Barnes (2) reported recently that 1 per cent of cobalt ( $\text{CoCl}_2$ ) added to the mixed diet of rats produced a polycythemia. It should be noted that the amounts of cobalt used by Waltner and Waltner (6) and by Myers, Beard, and Barnes (2) were significantly larger than that given by us.

It should be emphasized that in our experiments all rats fed a milk-iron diet supplemented by *cobalt without copper*, using either a curative (5) or a preventive (4) procedure, have developed an *anemia* and have died, whereas those given the milk-iron diet supplemented by *cobalt with copper* have developed a *polycythemia*. These results add to the evidence that copper is an essential factor in hemoglobin and erythrocyte production, and, furthermore, lead us to conclude that copper in the diet is necessary for the development of a cobalt polycythemia. Consequently, we believe that Myers, Beard, and Barnes (2) and Waltner and Waltner (6) were successful in producing a polycythemia with cobalt, only because the stock diets used by them contained sufficient copper to permit the development of the polycythemia.



The mechanism involved in the production of the polycythemia when cobalt is administered is not known; however, we believe that dehydration is not a factor. Although low body weights were observed in the animals receiving either  $\text{CoCl}_2$  or  $\text{CoSO}_4$ , no evidence of dehydration was found. The low weights may be explained by the fact that these animals did not consume as much milk as did those of the non-cobalt groups; however, when a cobalt animal ate exceptionally well and attained a comparatively high body weight, the polycythemia became even more pronounced. Further studies to determine the possible nature of the condition are in progress.

#### SUMMARY

1. Cobalt (0.5 mg.), as  $\text{CoCl}_2$  or  $\text{CoSO}_4$ , when fed *with copper* as a supplement to a milk-iron diet, produces a polycythemia in young rats.
2. Hemoglobin, erythrocyte, and cell volume values show a parallel rise, while the leucocyte and differential leucocyte counts show no significant alteration.
3. The polycythemia persists as long as cobalt is continued as a supplement with copper and iron to a whole milk diet.

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# A STUDY OF THE BLOOD GLUTATHIONE

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Since the discovery and isolation of glutathione from tissues by Hopkins (7) several methods have been elaborated for its quantitative determination in tissue extracts. The basic principle of all the methods in common use is the oxidation of the sulfhydryl group,  $-SH$ , to the disulfide group,  $S-S$ , by a measurable amount of oxidant. Obviously these methods include the determination of sulfhydryl compounds other than glutathione, but since this substance is present in relatively large amounts, the results are preferably expressed in terms of it. Thioneine, for instance, which is also a sulfhydryl compound, is present in blood in quantities of 4.2 to 15 mg. (1). Free cysteine is apparently absent from blood (6). The amount of glutathione, on the other hand, has been variously estimated by different investigators, the values obtained by different methods ranging from 20 to 90 mg. per 100 cc. of blood. These widely differing figures seem to indicate that there are variations between the methods used. Without recovery experiments the reliability and validity of a method appear questionable. In a recent paper Gabbe (4) has given an interpretation of the different results obtained by three different methods applied to one and the same specimen of blood.

The present study is concerned first, with qualitative tests of a large number of blood filtrates and, second, with quantitative determination of reduced glutathione with a colorimetric method recently published by Mason (9).

## EXPERIMENTAL

### *Qualitative Tests*

50 Folin-Wu blood filtrates and 50 10 per cent trichloroacetic acid filtrates were qualitatively tested with sodium nitroprusside

for the presence of free sulfhydryl groups. All of the 100 filtrates, the amount of which varied from 1 to 5 cc., gave a positive test; the trichloroacetic acid filtrates developed a much fainter color than the Folin-Wu filtrates.

Recently Feigl (3) has found a very sensitive test for free —SH ions. In the reaction,  $2\text{NaN}_3 + \text{I}_2 = 2\text{NaI} + 3\text{N}_2$ , —SH ions are the catalyst. This reaction can be applied to aliphatic sulfhydryl compounds of the type of cysteine, whereas cyclic compounds, as for instance thiophenol and thiocresol, fail to give a positive reaction. For small amounts of —SH groups the test is carried out advantageously as follows: To the solution to be tested add about 0.1 gm. of sodium azide and the same amount of finely powdered potassium iodide. Shake well and from a burette drop 1 to 2 cc. of 0.1 N iodine solution into the mixture. In the presence of free sulfhydryl groups an immediate formation of nitrogen gas takes place, which is very easily recognized even in very small amounts. When the test is applied to a trichloroacetic acid blood filtrate, the solution has to be neutralized with sodium carbonate to a pH of approximately 6. 50 Folin-Wu filtrates and 50 trichloroacetic acid filtrates were tested in this way. All of them showed positive reactions.

#### *Quantitative Determinations*

An adaptation of the colorimetric method of Mason (9) was used.

*Principle of Method*—The sulfhydryl groups are oxidized to disulfide groups in a buffered solution at pH 5.9 with potassium ferricyanide. The ferrocyanide formed is converted to Prussian blue with ferric sulfate and the color produced is compared with the color of a cysteine standard treated in the same manner.

Herbert, Bourne, and Groen (5) showed that all of the glutathione added to blood plasma (which itself does not contain sulfhydryl groups) passes into the Folin-Wu tungstic acid filtrate. This was confirmed in this laboratory. Recovery experiments on serum and whole blood are recorded in Table I.

Since glutathione of a high quality has been placed on the market it appeared logical to use this substance as standard. The purity of the product can be ascertained by titration with 0.01 N potassium ferricyanide at pH 7.4 as described by Mason (9). A

standard stock solution was prepared by dissolving 100 mg. in 5 cc. of 5 N sulfuric acid and diluting to 100 cc. with distilled water. This solution does not deteriorate for at least 3 months when kept in the ice box. The working standards contain 1 and 0.5 cc. respectively of the stock standard. For the preparation of the special reagents Mason's (9) paper must be consulted.

The determination was carried out in accord with Mason's directions. 20 cc. of a Folin-Wu tungstic acid blood filtrate in a 1:5 dilution were used (2 volumes of water instead of 7 volumes as in the original method), the final volume being 50 cc.

TABLE I  
*Recovery of Reduced Glutathione Added to Serum and Blood*

Serum			Blood		
Glutathione added to 100 cc	Glutathione found per 100 cc.	Glutathione recovered	Glutathione added to 100 cc.	Glutathione found per 100 cc.	Glutathione recovered
mg.	mg.	mg.	mg.	mg.	mg.
10	10 0	10 0	0	16.2	
20	19 6	19 6	10	26 6	10 4
30	31 1	31 1	20	37.1	20.9
40	39 8	39.8	30	46 1	29.9
			40	55 8	39.6

*Calculation*—When the stronger standard, containing 1 cc. of the stock solution, is used the following relation holds

$$\frac{\text{Reading of standard}}{\text{Reading of unknown}} \times 25 = \text{mg. of reduced glutathione per 100 cc. of blood}$$

For the weaker standard, containing 0.5 cc. of the stock solution, the equation is as follows:

$$\frac{\text{Reading of standard}}{\text{Reading of unknown}} \times 12.5 = \text{mg. of reduced glutathione per 100 cc. of blood}$$

*Blank*—Mason (9) found that the blank in which formaldehyde prevents the oxidation is zero for blood. This finding was confirmed many times. Therefore a blank is not necessary for glutathione determinations in blood with this method.

*Normal Blood*—In Table II the figures for reduced glutathione of twenty-one persons, ten males and eleven females of different age, are given. These blood specimens showed normal figures for sugar and non-protein nitrogen. The amount of reduced glutathione ranges from 14.7 to 38.8 mg. per 100 cc. of blood. The highest figure is thus well below those of 50 to 100 mg. which are found in the literature.

*Sulphydryl Groups of Venous and Arterial Blood*—Blanchetière, Binet, and Mélon (2) found that the amount of reduced glutathione

TABLE II  
*Blood Glutathione in Normal Persons*

Males				Females			
Subject	Age	Non-protein N	Gluta-thione	Subject	Age	Non-protein N	Gluta-thione
	<i>yrs.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>		<i>yrs.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>
J.W.	23	26.2	20.2	L.C.	20	27.0	16.1
F.C.	27	29.4	26.3	E.L.	26	28.2	18.5
P.McC.	32	33.3	24.3	M.S.	29	31.2	16.8
E.L.	40	27.7	30.7	A.L.	33	34.6	25.0
H.M.	46	32.6	29.9	M.McC.	35	32.7	17.3
F.LeJ.	48	27.5	15.2	R.M.	40	29.4	23.4
L.Sh.	59	36.1	16.6	M.J.	43	27.5	30.7
W.S.	62	28.7	18.3	J.P.	48	31.9	17.6
F.N.	63	35.1	16.5	S.F.	57	38.9	38.8
J.W.	69	36.1	14.7	E.H.	67	29.7	24.4
				H.B.	74	34.6	21.2
Average....		31.4	21.3	Average.....		31.4	22.7

thione in arterial blood from the carotid was lower than that of the venous blood from the right side of the heart. These investigators concluded that the reduced glutathione decreases when the blood passes through the pulmonary system. The method of determination used was the iodometric titration in a trichloroacetic acid filtrate according to Tunnicliffe (11).

A similar study of the sulphydryl groups of venous and arterial blood in the systemic circulation of dogs was made as follows: 10 to 12 cc. of venous blood were collected from the leg vein and the same amount of arterial blood from the femoral artery of the fast-

ing animal. Simultaneous determinations of reduced glutathione were carried out. The results are summarized in Table III. The values for the two kinds of blood change, and the lower figures are not exclusively confined to the arterial blood. It seems that the differences depend on factors other than oxygenation.

*Aeration*—By means of aeration of venous blood Gabbe (4) demonstrated a decrease of the sulfhydryl groups. The blood

TABLE III

*Comparison of Glutathione Content of Venous and Arterial Blood in Systemic Circulation of Dogs*

Sample No.	Glutathione	
	Venous blood	Arterial blood
	mg. per 100 cc.	mg. per 100 cc.
1	17.7	22.1
2	23.8	18.7
3	17.6	18.8
4	13.2	21.2
5	20.5	24.0
6	14.5	11.2

TABLE IV

*Influence of Aeration of Blood on Amount of Reduced Glutathione*

Sample No.	Aeration time	Glutathione	
		Before aeration	After aeration
	min.	mg. per 100 cc.	mg. per 100 cc.
1	2	26.9	23.6
2	5	23.4	13.4
3	8	23.9	10.3
4	10	24.5	7.85
5	12	23.4	6.61

filtrates this author used were prepared with trichloroacetic acid. Estimations in Folin-Wu filtrates did not show differences. We could not confirm these findings, for aeration of venous blood decreased the sulfhydryl groups estimated in the Folin-Wu filtrates. The decrease, as Table IV demonstrates, is roughly proportional to the aeration time. The procedure used was as follows: 25 cc. of venous blood from a dog were collected under

oil. 10 cc. were used for an immediate glutathione determination. The rest was transferred to a 2 liter separatory funnel and aeration by continuous rotation was carried out; then a determination was made.

*Oxidation with Hydrogen Peroxide*—A complete oxidation of the sulfhydryl groups was obtained with hydrogen peroxide. 10 cc. of blood were cooled in ice and 0.3 cc. of 30 per cent hydrogen peroxide (superloxol, Merck) was cautiously added, drop by drop. The mixture remained in ice for 30 minutes. In five specimens no color was obtained in the Folin-Wu filtrates; the qualitative test with sodium nitroprusside was also negative.

TABLE V  
*Influence of Reduction of Folin-Wu Filtrates with Nascent Hydrogen on Sulfhydryl Groups of Blood*

Sample No.	Glutathione	
	Before reduction	After reduction
	mg per 100 cc.	mg per 100 cc.
1	17 0	28 7
2	25 2	29.3
3	18 2	33 1
4	16 9	34 2
5	18 7	31 4
6	19 9	24 2
7	18.9	45 1

*Reduction*—The question is of interest whether all of the glutathione in blood is present in the reduced form or whether a part is oxidized which, on reduction, raises the values of determination. Thompson and Voegtlin (10) hold that nearly all of the glutathione is present in the reduced form, for they could demonstrate an increase of about 5 per cent on reduction. No details of the technique used are given. Likewise, Hess (6) obtained an average increase of 5 per cent by reducing blood filtrates with zinc and hydrochloric acid. Recently Kühnau (8) cast doubt on this mode of reduction. This author used sodium cyanide for this purpose. After trying out this salt in the present colorimetric method it had to be abandoned, for on standing it interfered with the color reaction. The following procedure was finally adopted.

To 25 cc. of a 1:5 Folin-Wu blood filtrate 5 drops of concentrated sulfuric acid were added followed by small portions of magnesium powder. After each addition the reaction was allowed to go to completion, and then the acidity was tested with Congo red paper. When the reaction became weak to Congo red, the solution was filtered and evacuated. In 20 cc. of the filtrate a determination of the sulfhydryl groups was carried out.

As it is shown in Table V in some cases the increase in sulfhydryl groups, as determined with the present method, is remarkable and amounts to over 100 per cent. However, from what is known on this subject it appears that this big increase can hardly be accounted for by originally oxidized glutathione. Experiments

TABLE VI

*Influence of Oxidation of Blood with Hydrogen Peroxide on Sulfhydryl Groups Determined after Reducing Folin-Wu Filtrate with Nascent Hydrogen*

Sample No	Glutathione	
	Original blood	Reduced Folin-Wu filtrate of oxidized blood
	mg. per 100 cc.	mg. per 100 cc.
1	24 5	23.5
2	16 7	16 3
3	32.1	33.8
4	25 8	22.5
5	30 9	30.4

to recover oxidized glutathione quantitatively from blood filtrates, subjected to the same reduction process, were unsuccessful, owing to the fact that this substance could not be obtained in pure form. Further investigations on this subject are in progress. At present it might be safe to assume that, when nascent hydrogen is employed for the reduction of oxidized glutathione in blood filtrates, other substances are also reduced which act upon ferricyanide at pH 5.9 and cause the high figures obtained. A possible clue to the solution of this problem was furnished by the following experiment. When blood is treated with hydrogen peroxide in the manner described before, the sulfhydryl groups are oxidized. The Folin-Wu filtrate of this blood, subjected to reduction with magnesium powder and sulfuric acid, gives a value for sulfhydryl



groups almost the same as the original blood. This seems to indicate that by oxidation these substances, which formerly, after reduction of the blood filtrate, acted upon ferricyanide at pH 5.9, are chemically so changed that their reducing power cannot be restored. The results are illustrated in Table VI.

#### SUMMARY

1. Mason's colorimetric method for the determination of reduced glutathione has been applied to blood.

2. The quantity of reduced glutathione in the blood of twenty-one normal persons ranged from 14.7 to 38.8 mg. per 100 cc., with an average of 21.3 mg. per 100 cc. in males and 22.7 mg. per 100 cc. in females.

3. The amount of sulfhydryl groups in venous and arterial blood from the same individual differs.

4. Aeration of blood decreases the sulfhydryl groups.

5. Oxidation of blood with hydrogen peroxide destroys all sulfhydryl groups.

6. Reduction of Folin-Wu blood filtrates with magnesium powder and sulfuric acid markedly increases the amount of substances which reduce potassium ferricyanide at pH 5.9.

7. The reduced Folin-Wu filtrate of blood oxidized with hydrogen peroxide shows a quantity of sulfhydryl groups almost equal to that of the original blood.

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## STUDIES ON KETOSIS

### I. THE SEXUAL VARIATION IN STARVATION KETOSIS\*

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Although numerous experiments have been carried out on fasting in the human, only a few of these have given a complete picture of the acidosis which develops during the early days of inanition. The data on the composition of the urine of normal women during fasting are especially limited. Because of the paucity of experimental evidence a comparison of the response of the two sexes to fasting has not been made.

While producing ketosis in the human to test the ketolytic action of various carbohydrates (1), it soon became evident that a distinct variation in the extent of the acidosis existed between the sexes. The amount of the acetone bodies excreted by the female subjects averaged about 3 to 4 times as much as in the males. While the male subjects were able to complete a 7 day fast with a minimum of physical discomfort, the women who served as subjects in many cases became so ill that they were confined to their beds and it was necessary to administer sugar to them at a much earlier time than was the case with the males. In the present paper, the record of these experiments is given only for the fasting days preceding the ingestion of the test meal of sugar. The data on the ketolytic action of sugar will be given in later papers.

\* A preliminary report of some of these data was given at the meetings of the Federation of American Societies for Experimental Biology at Montreal in April, 1931.

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## EXPERIMENTAL

*Methods*—Fasting was generally continued for a period of 7 days except in those cases where the subject became so ill that it seemed wise to terminate the fast earlier. During this time no food was eaten except on the morning of the 5th day when the sugar to be tested was taken in lemon juice. Water was allowed *ad libitum* and in some cases black unsweetened coffee or tea.

TABLE I  
*Acetonuria during Early Days of Fasting in Female*

Subject	Fast No.	Height	Weight	Surface area	Acetonuria, gm. per sq m.				
					Control	1st day	2nd day	3rd day	4th day
		in.	lbs.	sq. m.					
R. B.	1	64	165	1.80	0.07	0.14	7.54	9.03	8.18
	2		157	1.77	0.02	0.07	3.03	10.84*	
	3†		148	1.72	0.02	0.15	7.05		
G. C. D.	1	62	131	1.60	0.02	0.03	2.51	12.92	7.57
	2		134	1.62	0.02	0.02	1.16	9.00	
	3†		140	1.64		0.05	3.04	9.62	
M. G.	1	57	100	1.35	0.00	2.17	4.47	4.96	7.30*
R. M.	1	66	124	1.63	0.05	0.34	4.03	6.11	6.24*
V. T.	1	64	147	1.71	0.01	2.49	5.45	9.43	6.60
	2		144	1.70	0.01	0.27	3.45	4.89	4.68
	3		147	1.71	0.00	0.21	4.96	7.89	5.32

\* Estimated from 8 hour period.

† Menstruation just preceding or during period of fast.

Five women and five men of undoubted honesty served as subjects in these experiments. In addition to the authors, these included graduate students, technicians, internes, and several others closely connected with the medical school. All were apparently normal individuals. The variation in stature of the subjects in both sexes was from thin to stout. In order to make a satisfactory comparison of the acetone excretion of the different members of the group, it has been computed on the amount per sq.m. of body surface. Urine was collected in 24 hour periods

beginning before breakfast at 7.00 a.m. on the morning of the control day preceding the fast. Toluene was used as a preservative. The analyses were usually made daily. Urinary nitrogen

TABLE II  
*Acetonuria during Early Days of Fasting in Male*

Subject	Fast No.	Height	Weight	Surface area	Acetonuria, gm. per sq.m.				
					Control	1st day	2nd day	3rd day	4th day
J. S. B.		in.	lbs.	sq.m.					
	1	70.5	150	1.86	0.04	0.04	0.05	0.28	0.37
	2		152	1.87		0.07	0.49	1.93	4.02
	3		154	1.88	0.00	0.00	0.27	2.16	3.30
	4		155	1.88	0.03	0.06	1.47	2.83	4.00
H. J. D.	5		156			0.05	0.79	2.36	3.96
	1	70.5	190	2.05	0.02	0.12	1.00	2.86	3.80
	2		186	2.04	0.02	0.14	2.46	3.95	4.38
	3		190	2.05	0.03	0.06	1.41	2.25	4.16
	4		194	2.07	0.02	0.01	1.16	4.03	3.13
	5		191	2.05	0.04	0.05	2.14	3.97	3.33
P. L.	6		199	2.09	0.03	0.15	2.43	3.48	4.28
	1	68	148	1.80	0.04	0.04	0.52	1.12	2.39
	2		151	1.82	0.02	0.01	0.17	0.77	1.06
P. W. S.	3		157	1.84	0.01	0.03	0.27	0.71	0.90
	1	68	137	1.74	0.04	0.02	0.03	0.06	0.16
	2		142	1.77		0.02	1.01	0.69	1.97
	3		144	1.78	0.00	0.00	0.71	1.79	1.92
	4		146	1.79	0.00	0.03	0.28	1.21	2.02
C. T.	5		142	1.77		0.02	0.84	2.18	2.74
	1	70	240	2.26	0.04	0.02	0.27	0.70	1.86
	2		240	2.26	0.01	0.10	0.81	4.60*	3.34*
	3		240	2.26	0.00	0.02	0.23	0.57	2.15

\* Hard manual labor during this experiment. Not included in average.

analyses were carried out by the usual Kjeldahl technique and acetone bodies were determined by the method of Van Slyke.

*Results*—Twenty-two experiments were made on five male subjects and eleven tests on five women. Tables I and II give the individual results in the experiments on the female and male

TABLE III

*Comparison of Average Acetonuria during Early Days of Fasting in Male and Female*

Day of fast	Male subjects		Female subjects	
	No. of experiments	Acetonuria gm.	No. of experiments	Acetonuria gm.
Control	18	0.02	10	0.02
1	22	0.05	11	0.54
2	22	0.86	11	4 25
3	21	1 90	10	8 47
4	21	2.66	7	6 56

TABLE IV

*Urine Nitrogen during Early Days of Fasting in Male*

Subject	Fast No.	Urine N, gm. per 24 hrs.				
		Control	1st day	2nd day	3rd day	4th day
J. S. B.	1	14.11	10.71	10 35	16 55	11 44
	2		10.38	9 97	11 44	10 86
	3	12.83	9.11	9.13	11.22	11 94
	4	10 89	9.59	9 83	13 17	12 70
	5	13.59	10.86	11 70	12 74	13 73
H. J. D.	1	11.82	8.89	9 90	12 69	11 97
	2	13.02	10 15	11 69	13 38	12.21
	3	8.88	10 02	11 27	11 52	11 20
	4	14.25	11 38	8 83	12.04	11 45
	5	13 10	11 53	11 22	12 84	11 72
	6	11.64	10 26	11 84	11 80	12 39
P. W. S.	1	15 68	11 09	10 88	7 98	12 50
	2		9 09	11.03	11 65	11 63
	3	14 02	9.77	11 10	12 84	10 69
	4	14.20	8 81	11 79	12.63	11 80
	5	12 51	8 48	10.12	11.73	12 31
C. T.	1	14 45	12 57	13.37	14.21	14 61
	2	13 45	11 27	11 57	12.40	10 61
	3	19 01	14.21	14 21	14.28	15.52

subjects, and Table III gives the comparison of the average results.

**TABLE V**  
*Urine Nitrogen during Early Days of Fasting in Female*

Subject	Fast No.	Urine N, gm. per 24 hrs.				
		Control	1st day	2nd day	3rd day	4th day
R. B.	1	8.65	4.68	10.60	11.71	10.66
	2	8.18	4.72	5.84	9.89*	
	3	6.86	6.22	10.73		
G. C. D.	1	8.95	6.13	7.39	9.44	
	2	7.61	5.52	5.80	8.29	
	3		7.42	7.46	9.79	
M. G.	1	16.52	10.43	11.62	9.86	
R. M.	1	12.19	7.94	12.25	11.58	
V. T.	1	12.11	9.81	11.91	9.91	9.00
	2	10.88	7.24	9.35	9.89	8.09
	3	13.17	7.38	12.18	10.85	9.26

\* Calculated on basis of 8 hour sample.

**TABLE VI**  
*Comparison of Average Urinary Nitrogen during Early Days of Fasting in Male and Female*

Sex	Urinary N, gm. per 24 hrs.				
	Control	1st day	2nd day	3rd day	4th day
Female.....	10.51	7.05	9.56	10.12	
Male.....	13.38	10.38	11.04	12.42	12.12

**TABLE VII**  
*CO<sub>2</sub>-Combining Power of Blood during Fasting*

Subject	Sex	Fast No.	CO <sub>2</sub> -combining power		
			1st day	3rd day	4th day
R. B.	Female	1	vols. per cent 53.6		26.9
		2	52.8	32.8	
		3	55.7	34.3	
P. L.	Male	1	52.1		46.6
		2	57.6		42.4
		3	58.5		51.7

Small but practically equal amounts of acetone bodies were excreted by the two groups of subjects on the control days. However, from the 2nd to the last day of the fast the women excreted greater amounts of acetone than the men, except for one female subject who, on the 2nd day, eliminated less acetone than the maximum excretion among the male subjects. The mean values show that about 10 times as much acetone bodies was excreted the 1st day, 5 times as much the 2nd day, 4 times as much the 3rd day, and more than 2 times as great an amount on the 4th fast day. It has been impracticable to continue the fasts sufficiently long to determine whether these would ultimately reach the same level.

The values for the urinary nitrogen for the same experiments are recorded in Tables IV to VI.

In harmony with many other investigators, we found a decreased nitrogen excretion on the 1st day of the fast. In all probability, this is due to the protein-sparing action exerted by the oxidation of the glycogen remaining in the liver. An increase in nitrogen excretion occurs in the case of the female subjects on the 2nd day. This is supposed to occur coincidentally with the exhaustion of the stored carbohydrate. In five experiments on male subjects, a further decrease in urinary nitrogen was found on the 2nd fast day. The average decline of the urinary nitrogen from the control day was 32, 9, and 4 per cent for the women and 22, 18, and 8 per cent for the men during the first 3 days of the fasts. The shorter period of decrease in urinary nitrogen for the women is in harmony with their more rapid susceptibility to acidosis.

The extent of the acidosis is indicated also by the fall of the  $\text{CO}_2$ -combining power of the blood. The results in Table VII show that the decrease was much more severe in each of the three experiments on R. B., a woman, than occurred in any of those on a male subject, P. L., who had fasted for the same length of time and who excreted only a comparatively small amount of acetone bodies in the urine.

#### DISCUSSION

The women subjects showed a much greater susceptibility to fasting ketosis than the male ones. This was indicated not only

by the more rapid development of the ketosis but also by the higher levels of acetonuria which obtain during the 3rd and 4th fast days. The maximum level of acetone excretion was reached in most cases with the women on the 3rd day, while a small drop in the elimination of acetone bodies usually took place on the 4th day. We have no data on whether a further decrease would take place if the fast were continued. The maximum value was reached in the men subjects at a later interval. In only three of the twenty-two experiments was the acetonuria higher on the 3rd than on the 4th fast day. The small extent of the increase in acetonuria from the 3rd to the 4th day as well as the results of two unpublished control experiments would seem to indicate that the peak of acetone excretion is reached on the 4th day. It seems possible that in the female the excretion might continue at a higher level than in the male during a fast longer than 4 days.

The greater severity of the acidosis in women was indicated also by the greater decrease in the  $\text{CO}_2$ -combining power of the blood during fasting in the female than in the male. In R. B., a female subject, the  $\text{CO}_2$ -combining power fell from the normal value to 26.9 volumes per cent on the 4th day of the fast while in two other experiments it had dropped to 32.8 and 34.3 volumes per cent on the 3rd fast day. A minimum value of 42.4 volumes per cent was obtained on a man, P. L., on the 4th fast day while in two other experiments the value had only dropped to 46.6 and 51.7 volumes per cent on the same day.

The conclusion of Folin and Denis (2) that obesity is not a predisposing cause for acidosis is borne out by the results here recorded. Subject C. T., who weighed 240 pounds and might be described as obese, produced only 1.59 gm. of acetone bodies on the 3rd day of the first fast in contrast with 5.32 gm. which were eliminated by J. S. B., who was a fairly thin subject, on the corresponding day of his fourth fast. An even greater discrepancy is shown when the above results are compared with those of G. C. D., a woman, who weighed only 131 pounds and who excreted almost 21 gm. of acetone bodies on the 3rd day of the first fast. Since the male subjects varied in stature from thin to obese while the women might be classified as thin to moderately fat, it becomes evident that obesity is not the cause of the difference observed between the sexes.



Although it does not seem that the amount of adipose tissue is the determining factor in the degree of acetonuria we have evidence suggesting that a relationship exists between the nutritional condition and the acetonuria. J. S. B. and P. W. S. undertook their first fast when they had taken a normal diet for only a week following the conclusion of a prolonged protein-fat diet on which each had lost considerable weight. During this fasting period the acetonuria was almost negligible. J. S. B. excreted a total of only 0.63 gm. on the 4th fast day while P. W. S. eliminated only 0.28 gm. on the same day. In later experiments at the beginning of which these subjects were in far better nutritional condition, the acetonuria on the 4th fast day amounted to about 7 gm. with J. S. B. and from 3 to 5 gm. with P. W. S. With the exception of these two cases, the men who served as subjects appeared to be in excellent condition so that the lower values found in males cannot be traced to nutritional differences at the start of the fasting period.

In practically all of our subjects, irrespective of sex, we have noted a drop in protein metabolism on the 1st and sometimes on the 2nd fast day. An increase invariably follows which is generally considered to occur when the glycogen reserves are largely exhausted. The average results indicate a somewhat more prolonged protein-sparing action in the male than in the female subjects. During the first 3 days of the fast the total decrease in urinary nitrogen below the level of the control day is 22, 18, and 8 per cent for the males and 32, 9, and 4 per cent for the female subjects. These results might indicate that a greater glycogen store exists in the male or that it is more economically used during the early days of the fast. If such were the case, one would have an adequate explanation for the readier development of the ketosis in the female subject. In support of this may be cited the experiments of Greisheimer (3) who reported that female rats invariably have a smaller glycogen store in the liver as well as a larger lipid content than do male rats similarly treated. After 48 hours fasting, the average glycogen content in the liver of male rats was 0.51 per cent while that of the female ones was only 0.13 per cent. It seems certain that the variation in the rate of development of the acidosis is not to be attributed to dietary differences preceding the fast. Although the diets were not fixed for the con-

trol day, all of the subjects were on a normal, mixed non-reducing diet. In three cases (H. J. D. and G. C. D., C. T. and V. T., P. L. and R. B.) the male and female subjects partook of the same food preceding the fast. The general uniformity in the level of the urinary nitrogen (6.83 gm. per sq.m. per day for the males and 6.53 gm. for the female subjects) offers further evidence for the constancy of the food intake in the two sexes.

There is no evidence from our experiments that a repetition of fasting habituates the organism to this condition so that a complete or more complete oxidation of the fats may occur. Folin and Denis (2) have drawn this conclusion from the results of subsequent fasts on two abnormally obese women. A progressive decline in the  $\beta$ -hydroxybutyric acid excretion occurred on the corresponding days of the different fasts. No such differences have been noted in any of our eight subjects of either sex who fasted three times or more. With H. J. D., who fasted six times during a period of 7 months, almost identical results were obtained on the last fast as were found on the first one. Thus, the elimination of acetone bodies during the first 4 days of the first fast was 0.12, 1.00, 2.86, and 3.80 gm. respectively per sq.m. of body surface, while the values for the corresponding days of the last experiment were 0.15, 2.43, 3.48, and 4.28 gm. Similar results were found on our other subjects. The discrepancy between these results and those of Folin and Denis may be due to the fact that their work was carried out on abnormally obese women or because of the poorer nutritional condition of their subjects at the start of the later fasts as the result of the small interval which elapsed between the experiments.

#### SUMMARY

1. A much greater ketosis develops during fasting in the female than in the male. This is indicated not only by the greater excretion of acetone bodies in the urine but by the greater fall in carbon dioxide-combining power of the blood.

2. A decrease in nitrogen excretion from the control level followed by a later increase occurs with both sexes. The decrease, which is supposed to be traced to the sparing action of the glycogen still remaining in the tissues, seems to be more prolonged with the male subjects.

3. In none of the eight subjects, on whom three or more fasts were carried out, is there any indication of a habituation to fasting. The acetone excretion on the last fasts were essentially of the same magnitude as those on the first ones.

4. Obesity is not a predisposing cause of acidosis. However, two subjects who started fasts in extremely poor nutritional conditions excreted only insignificant amounts of acetone bodies as compared with the later fasts which they began in a far better nutritional condition.

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## CAROTENE

### IV. THE HYDROGENATION OF CAROTENES OBTAINED FROM DIFFERENT SOURCES, OF DIHYDROCAROTENE, AND OF LYCOPIN

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#### INTRODUCTION

The accurate determination of the number of mols of hydrogen which can be added catalytically to an organic substance is most important in distinguishing between the different types of unsaturation present in its molecules. By such a determination it is possible to differentiate between double bonds and rings in unsaturated hydrocarbons. For this reason observations on the catalytic hydrogenation of carotene and related substances have been continued.

The last article of this series (9) dealt with the hydrogenation of carotene from carrot roots. In the experiments there reported it was found that 10 mols of hydrogen were absorbed per formula weight of carotene hydrogenated; *i.e.*, it was found that carotene from carrot roots possessed a hydrogenation ratio of 10.<sup>1</sup> At that time it was thought that a greater hydrogenation ratio might be obtained if a larger proportion of catalyst to carotene were used.

Work has now been completed in which the weight of catalyst has been increased to more than 5 times the weight of the carotene, but the hydrogenation ratio previously reported has not been altered.

These observations have been extended to the carotenes from

<sup>1</sup> By the term hydrogenation ratio is meant the number of mols of hydrogen absorbed per formula weight of substance hydrogenated.

leaves of several species of plants and the results show that these carotenes, too, have a hydrogenation ratio very close to 10.

In the present paper will be described the processes used for the preparation of the pigments, the micro method developed for the hydrogenation, and some of the conclusions which may be drawn from the study of the hydrogenation of these substances.

#### EXPERIMENTAL

##### *Carotene from Carrot Roots<sup>2</sup>*

Batches of approximately 32 kilos of fresh carrot roots (*Daucus carota*, L.) were washed and then cut into slices 4 mm. thick. The slices were freed from water-soluble material by two extractions with water, each for 2 hours at 50–60°. The carrot slices were then spread on trays, placed in a specially constructed dehydrator, and dried in a warm current of air at 50–60° for 22 hours. The dried chips were ground in a power grinder so as to pass a 20 mesh sieve, then pulverized in a pebble mill until the powder passed a 60 mesh sieve. The powder (1.67 kilos approximately) was steeped, at room temperature, in 1.75 liters of petroleum ether (b.p. <55°) for 1 hour with frequent stirring. The pulp was caught on a Buchner filter and washed with 500 cc. portions of petroleum ether until the washings were almost colorless. Usually four or five such washings were sufficient. The entire extract was centrifuged to remove any solid particles which passed the filter. The petroleum ether solution was concentrated to 300 cc. under reduced pressure. During the concentration of the solution, the temperature of the liquid never exceeded 35°. A flocculent precipitate always separated at this point. This material was removed by centrifuging the concentrated solution while it was yet warm. This solution was set in the refrigerator and the carotene allowed to crystallize.

After 48 hours the carotene crystals were collected in a centrifuge tube, washed with three 30 cc. portions of petroleum ether (b.p. <50°), dissolved in 30 cc. of chloroform, and the solution filtered by suction through a well packed asbestos mat in a Gooch crucible. The filtrate was poured into an evaporating dish, 10

<sup>2</sup> This method was developed by Harold W. Milner in collaboration with the author.

cc. of ethyl alcohol added, and the dish set over a vessel containing 100 cc. of ethyl alcohol. Both vessels were placed in a bell jar vacuum desiccator. The desiccator was evacuated to about 26 cm. pressure and then set aside. By interdiffusion of the two liquids, through the vapor phase, the carotene was precipitated from the chloroform solution in large flaky crystals. These crystals were filtered off, dried in a vacuum, and stored in evacuated ampoules. The average yield of recrystallized carotene per run was approximately 1.4 gm.

The authors are indebted to several publications for the general method here outlined. The chief sources are the methods of Kohl (6), Escher (2), and Schertz (8).

#### *Purification of Carotene for Hydrogenation<sup>3</sup>*

Numerous recrystallizations were made in an attempt to separate carotene from carrot roots into high and low melting fractions. Neither repeated recrystallizations from petroleum ether nor from pyridine yielded a fraction melting above 178°. However, leaf carotene, which melted above this temperature, could be obtained by a single recrystallization. A similar observation has been made by Kuhn (7).

The carotene from carrot roots, used for hydrogenation, was gotten by combining the carotenes obtained from the mother liquors of these recrystallizations. The combined carotenes were extracted with small quantities of boiling ethyl acetate and then isoamyl acetate. The residue, melting at 171.5–173.5°, was dissolved in pyridine, the solution filtered, and then diluted with ethyl acetate and methyl alcohol, while still warm. On cooling, the clear warm solution deposited crystals of carotene which melted at 172.7–174.2°. These crystals were analyzed and hydrogenated (*cf.* Table III, Experiments 1 and 7, Table IV, Experiment 10).

#### *Leaf Carotene<sup>4</sup>*

The method of Willstätter and Mieg (12) was used in modified form for the preparation of the leaf carotenes.

<sup>3</sup> Carried out by Harold H. Strain.

<sup>4</sup> This method was developed by Henry M. Leicester and Harold W. Milner.

Enough fresh green leaves to yield approximately 1.5 kilos of dried material were dehydrated at 50–60° for 22 hours. The dried material was ground and the powder steeped in 2 to 2.5 liters of petroleum ether (b.p. 40–70°) for about 16 hours. After filtering the extract, the leaf powder was washed with petroleum ether until the washings ran almost colorless. The combined extract and washings were shaken with 30 gm. of potassium hydroxide in 200 cc. of methyl alcohol to remove the chlorophyll, then washed with four 500 cc. portions of water. The petroleum ether solution was dried over sodium sulfate, concentrated to 100 to 200 cc., and double or treble its volume of absolute ethyl alcohol added. The addition of the alcohol precipitated a considerable quantity of fats and waxes which were immediately removed by filtration. The filtrate deposited crystals of carotene on standing in the refrigerator for 24 to 48 hours. This carotene was contaminated with fats and waxes which were partially removed by gently boiling the sludge with 25 cc. of petroleum ether (b.p. <50°) and filtering immediately. The carotene crystals which remained on the filter were then dissolved in chloroform and the pigment precipitated from the chloroform solution by the addition of absolute ethyl alcohol. This treatment was followed by a recrystallization from petroleum ether.

The carotenes from alfalfa leaves (*Medicago sativa*, L.) and from cauliflower leaves (*Brassica oleracea*, L., var. *botrytis*, L.) were analyzed and hydrogenated at this stage of purification.

The carotene from spinach leaves (*Spinacia oleracea*, L.) was given one more crystallization from petroleum ether, the crystallization occurring at about 4°. The crystals were dried in a vacuum desiccator.

The carotenes from fig (*Ficus carica*, L., var. *hortensis*), chard (*Beta vulgaris*, L., var. *cicla*, L.), and sunflower leaves (*Helianthus annuus*, L.) were prepared in the same manner as carotene from alfalfa leaves, then recrystallized from petroleum ether (b.p. 65–70°) at the temperature of solid carbon dioxide in ether. The crystals were collected and dried in an Abderhalden vacuum drying apparatus for 45 minutes at the temperature of boiling methyl alcohol. These carotenes were stored in a vacuum desiccator for 1 or 2 days until analyzed and hydrogenated.

Carotene was obtained from sugar-beet leaves (*Beta vulgaris*,

L., var. *rapa*, Dumort.) in the same manner as from alfalfa. This carotene was dissolved in petroleum ether (b.p. 65–70°) and shaken with a small quantity of fullers' earth. After removing the fullers' earth, the solution was concentrated and set in the refrigerator. After about 48 hours the crystals which separated were collected, dried in a vacuum desiccator, then analyzed and hydrogenated.

In the present state of knowledge of the structure of carotene it is essential that a complete history of the carotene be known. For this reason the methods of preparation and purification have been described in detail for all the samples of carotene used in this investigation.

TABLE I  
*Yield of Carotene from Various Leaves*

Experiment No.	Kind of leaf	Fresh weight	Dry weight	Carotene	
		kg	kg.	gm.	per cent dry wt.
1	Black fig	27 80	7.43	0.18	0.0024
2	Sugar-beet	125 97	17.34	0.26	0.0015
3	Chard	186 15	17.71	0.97	0.0054
4	Spinach	183.56	18.52	1.61	0.0087
5	Alfalfa	40.78	9.29	0.16	0.0017
6	Cauliflower	47.30	7.22	0.24	0.0033
7	Sunflower	90 71	20.03	2.22	0.0111

In sunflower and spinach much more carotene was obtained from the younger than from the older plants.

In passing it is interesting to note the yields of carotene from different kinds of leaves. In Table I are given the fresh weight used, the dry weight, the weight of carotene obtained, and the per cent of carotene in the dry leaf material.

It has been the experience in this laboratory that there is little relation between the depth of color of the extract and the amount of carotene which can be obtained from it. It is hoped that a quantitative study of this relation may be made at some later time.

*Dihydrocarotene*<sup>5</sup>—Dihydrocarotene was prepared by reducing carotene in ether solution with aluminum amalgam. The method

<sup>5</sup> This was prepared by Harold H. Strain.



has already been described ((9) p. 598). The carrot carotene from which it was derived was part of the same sample for which hydrogenation and analytical data are given in Table IV, Experiment 10. That the carotene was reduced to the dihydro stage by the aluminum amalgam treatment is attested to by the analytical data, which are in better agreement with the empirical formula  $C_{40}H_{58}$  than with the formula for tetrahydrocarotene,  $C_{40}H_{60}$ .

$C_{40}H_{58}$ .	Calculated.	C 89.14, H 10.86, mol. wt. 538.5
	Found.	" 89.14, 89.04; H 11.04, 10.90; mol. wt. 557
$C_{40}H_{60}$ .	Calculated.	" 88.81, H 11.19, mol. wt. 540.5

The molecular weight was determined in benzene solution by the micro-Menzies-Wright method (11).

*Lycopin*<sup>6</sup>—Lycopin was prepared by a method which is very similar to that used by Escher ((2) p. 89).

The contents of six cans of puree of tomato (*Lycopersicum esculentum*, Mill.), which weighed about 18 kilos and contained 270 gm. of dry material, were poured into a large cloth bag and allowed to drain overnight. The pasty mass from this treatment was pressed in a screw press as free from water as possible and then dehydrated by soaking for about 16 hours in synthetic methanol. The solid matter was filtered off and pressed. After another like dehydration the pigment was extracted from the press-cake with carbon bisulfide. For this extraction the press-cake was broken up and stirred into enough carbon bisulfide to make a thin gruel and allowed to stand overnight. Usually two such extractions were enough to remove all the color. After removing the solid matter by filtration the extract was concentrated to 100 to 200 cc. and 200 to 300 cc. of absolute ethyl alcohol were added. The red sludge, which separated after standing in the refrigerator for 24 hours, was collected on a filter, dissolved in chloroform, and reprecipitated with absolute ethyl alcohol. The lycopin, obtained by this treatment, was crystallized from petroleum ether (b.p. 65–70°). From this solvent it separated in fine needle-like crystals which melted at 166.7–167.8°. About 0.14 gm. of lycopin was obtained in each run. Seven such runs were made.

<sup>6</sup> This was prepared by Henry M. Leicester.

The analyses and hydrogenation ratios of this material are shown in Table IV, Experiment 11, and Table V, Experiment 7.

Since the analyses of the lycopin obtained by the procedure described were none too good, some of the lycopin was further purified.

The lycopin was dissolved in chloroform, the solution filtered, and absolute ethyl alcohol added. The lycopin which precipitated was filtered off, dissolved in petroleum ether, and treated with a small quantity of fullers' earth. The fullers' earth was removed and the solution concentrated. Lycopin was crystallized from the concentrated solution by cooling the solution in an ice-salt mixture. These crystals were collected, washed with petroleum ether (b p.  $< 50^{\circ}$ ), and dried in a vacuum desiccator for 3 hours. The lycopin melted at  $166.7^{\circ}$ . The analyses gave C, 88.89 and H, 10.32 per cent. The theoretical values for  $C_{40}H_{56}$  are C, 89.48 and H, 10.52 per cent.

The mother liquor from the above crystallization was concentrated and set in the refrigerator. Lycopin crystals separated. These were collected on a filter, washed with petroleum ether, and dried in an Abderhalden vacuum drier at the temperature of boiling methyl alcohol. The crystals melted at  $167.9$ – $169.0^{\circ}$  and showed a composition of C, 89.29 and H, 10.22 per cent. This lycopin was hydrogenated (*cf.* Table V, Experiment 6).

*Preparation of Solvents*—The materials which were used in this investigation were all freshly prepared or purified in this laboratory.

The *acetic acid* was purified by recrystallization.

The *cyclohexane* had been recovered from previous hydrogenations of carotene; therefore it was completely saturated with hydrogen. Its boiling point,  $80.5$ – $80.8^{\circ}$ , indicated that it was good material.

*p-Menthane* was prepared by the hydrogenation of *d*-limonene. The hydrogenation was carried out in acetic acid solution with platinum oxide as the catalyst. After hydrogenation, the acetic acid was washed out with water and then with dilute sodium hydroxide solution. Any unsaturated hydrocarbon which remained was removed by washing with concentrated sulfuric acid. After this the *p*-menthane was washed with water, dilute sodium hydroxide solution, and then water again, dried over sodium sulfate, and finally distilled in a vacuum.

The *di-n-butyl ether* was obtained from Dr. Carl Noller of the Chemistry Department of Stanford University, in whose laboratory it was prepared. The ether distilled between 141–142° at 764.4 mm. pressure. We are indebted to Dr. Noller for giving us this material.

To prepare the solvent used in hydrogenation this ether (37 cc.) was mixed with glacial acetic acid (10 cc.) and saturated with hydrogen at 3 atmospheres pressure with platinum oxide catalyst. The mixture was decanted from most of the platinum black and stored in a glass-stoppered bottle. For each hydrogenation in this solvent, 5 cc. of this solution were used.

*Reference Substances*—The substances used to test the method of hydrogenation were *crotonic acid* (m.p. 72.3°), *sorbic acid* (m.p. 132.6–133.6°), *naphthalene* (m.p. 80.5°), and *stilbene* (m.p. 124.0–124.5°). The melting points are given to indicate the degree of purity of the material. These and all subsequent melting points reported in this paper were taken in a Berl melting point apparatus (1) and are corrected.

#### *Apparatus and Procedure*

By describing the method of procedure, the diagrams of the micro hydrogenation apparatus, shown in Figs. 1 and 2, will be made clear.

A pellet of the material to be hydrogenated was weighed on a micro balance and dropped into the tube *t*, Fig. 2. This tube was drawn out to a shape *t'* to give the added length necessary to prevent the solvent from splashing into the tube during the saturation of the catalyst and solvent.

The catalyst was then weighed into reaction bulb 1, Fig. 1 (capacity of bulb 34 cc.; capacity of reaction flask 61 cc.). The proper quantities of solvent were then added and the sample tube *t'* cautiously slid to the bottom of the bulb. Reaction flask 1 was attached to the apparatus through the slightly greased ground-glass joint *G*. The compensation flask, 2, loaded with the same amount and kind of solvent as flask 1, was likewise attached to the apparatus through the slightly greased joint *G'*. Stop-cock *s* was opened. Shaking of apparatus *A* on a mechanical shaker was then begun. The glass spring *C* and the long 1 mm. glass tube *F* allowed the reaction vessel *A* to be shaken with considerable amplitude.

After closing the screw clamp *p* the whole apparatus was evacuated through stop-cock *c*, cocks *d*, *e*, *f*, and *s* all being open, the others closed. *c* was then shut and hydrogen admitted

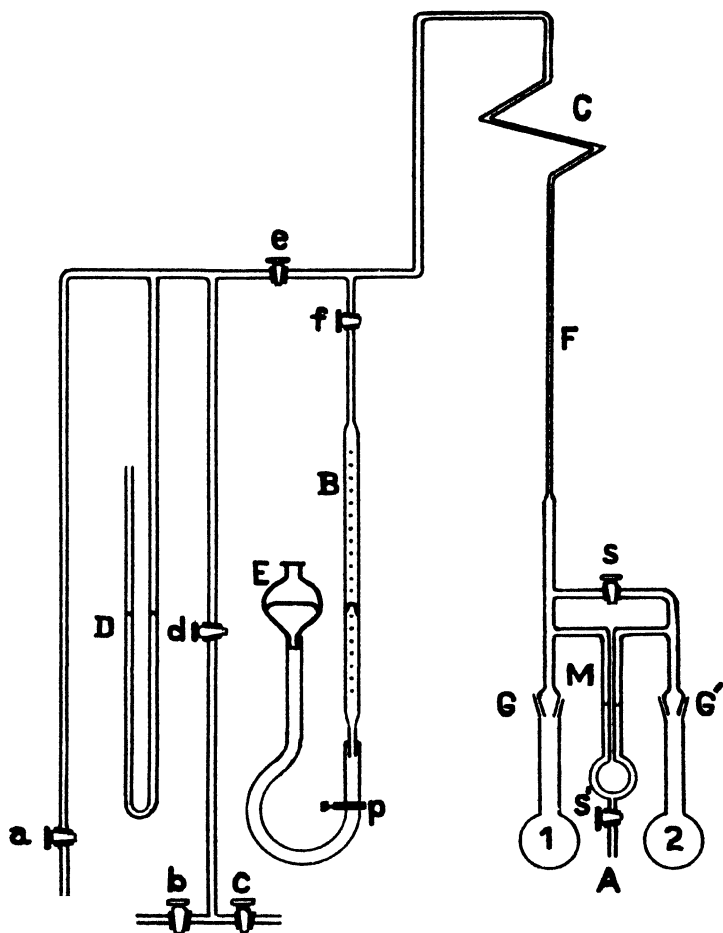


FIG. 1. Diagram of apparatus used for micro method of hydrogenation

through *b* until a slight excess pressure was registered on the mercury manometer *D*. The platinum oxide catalyst was reduced and the apparatus alternately evacuated and filled with hydrogen about ten times. Finally after filling with a slight excess pressure

of hydrogen the catalyst and solvent were completely saturated with hydrogen. This required about 40 minutes. Stop-cock *a* was opened to bring the apparatus to atmospheric pressure and the barometric pressure observed. Screw clamp *p* was then removed and all the stop-cocks but *f* closed. If the catalyst and solvent in flask *I* were not saturated, a change in pressure was registered on the *n*-butyl phthalate manometer *M*, when stop-cock *s* was closed, and the pressure was then equalized by changing the height of leveling bulb *E*. When no more hydrogen had



FIG. 2. Tube for introducing sample

been absorbed for approximately 30 minutes a reading of the volume in burette *B* was made and the thin bulb on the toe of sample tube *t'* broken by vigorously shaking the absorption apparatus *A*. The amount of hydrogen absorbed was followed by observing the change of volume in *B* necessary to keep the two levels in manometer *M* equal. When the volume reading had been constant for 30 minutes or more, the hydrogenation was assumed to be completed. The liquid used in *EB* was mercury.

The number of mols of hydrogen absorbed was calculated from the change in volume, the initial barometric pressure, and the

final temperature. From the weight of sample and the formula weight the number of mols of substance was obtained. Dividing the first by the second gave the *hydrogenation ratio*.

### Results

First, known compounds were hydrogenated in glacial acetic acid solution. The results of these determinations given in Table II showed that the method was accurate and reliable, the

TABLE II

*Hydrogenation of Known Compounds. Solvent, 5 Cc. of Glacial Acetic Acid*

Experiment No.	Substance			Catalyst	Barometric pressure	Temperature	H <sub>2</sub> absorbed		Hydrogenation ratio		Error
									Observed	Calculated	
		mg.	$\frac{mols}{\times 10^5}$	mg.	mm.	°C.	cc.	$\frac{mols}{\times 10^5}$			per cent
1	Crotonic acid	4.507	5 236	5 144	759 2	19.9	1 280	5 315	1 015	1 00	1.50
2	Naphthalene	2 023	1 579	6 661	759 2	19 0	1 880	7 836	4.962	5 00	0 77
3	Sorbic acid	2.890	2 579	4 685	753 4	20 2	1 255	5 166	2 003	2.00	0.15
4	Stilbene	1 809	1 004	10 770	757 9	20 2	1 715	7.102	7 073	7 00	1 05
Average error ...											0.86

average error being 0.86 per cent and the greatest error 1.50 per cent.

Carotene could not be hydrogenated readily in glacial acetic acid because of its relative insolubility in this solvent. It had been shown previously ((9) p. 602) that it could be hydrogenated easily in a mixture of cyclohexane and acetic acid, so this solvent was tried. These hydrogenations were carried out in a mixture of 3 cc. of acetic acid and 3 cc. of cyclohexane. The results are given in Table III.

The hydrogenation ratio for carotene in this solvent agreed well with that reported by Zechmeister and Cholnoky (13) in

cyclohexane solution. When, however, the hydrogenation ratios of known compounds were determined in cyclohexane-acetic acid, the values were found to be too high. The values for stilbene and

TABLE III  
*Hydrogenation in Solvent*

Experiment No.	Substance	Catalyst	Barometric pressure	Temperature	H <sub>2</sub> absorbed	Hydrogenation ratio		Error		
						Observed	Calculated			
3 cc. cyclohexane and 3 cc. glacial acetic acid										
1	Carotene from carrot roots	mg.	$\frac{\text{mols}}{\times 10^4}$	mg.	mm.	°C.	cc	$\frac{\text{mols}}{\times 10^4}$		per cent
		2.901	0.5407	17.532	758.2	21.0	1.435	5.929	10.96	
2	Stilbene	2.062	1.144	15.398	761.0	20.12	1.110	8.776	7.67	7.009
3	Crotonic acid	4.137	4.807	15.370	762.5	21.31	1.250	5.188	1.079	1.007
Average error.....										8.75
3 cc. <i>p</i> -methane and 3 cc. glacial acetic acid										
4	Crotonic acid	3.617	4.202	16.183	762.7	22.0	1.030	4.266	1.015	1.001
5	Sorbic acid	2.397	2.139	13.547	759.2	20.0	1.040	4.317	2.018	2.000
6	Stilbene	2.108	1.170	11.662	759.8	21.02	2.010	8.322	7.113	7.001
7	Carotene from carrot roots	2.727	0.5083	17.214	757.5	21.11	1.245	5.137	10.10	
Average error.....										1.34

crotonic acid were 9.6 and 7.9 per cent too high, respectively. This indicated that the hydrogenation ratio for carotene obtained in this solvent mixture was too high also. When the value for

carotene, 10.96, was divided by 1.088, the average correction factor found for stilbene and crotonic acid in this solvent mixture, a hydrogenation ratio of 10.1 was obtained. This is in entire agreement with the value for carotene reported previously (9) p. 602, (10)) from this laboratory.

On the assumption that the relatively high vapor pressure of cyclohexane (3) was responsible for these high values, a solvent with much lower vapor tension was chosen, *p*-menthane. The results obtained when a mixture of 3 cc. each of *p*-menthane and glacial acetic acid were used as solvent are reported in Table III.

As may be observed, the results for known compounds in the *p*-menthane-glacial acetic acid are satisfactory. The hydrogenation ratio for carotene was found to be 10.1. This value is in entire accord with the corrected value obtained in the cyclohexane experiment. The carotene used in these two experiments was taken from the same lot.

After this method for determining hydrogenation ratios with small quantities of material had been shown to be accurate and also applicable to the hydrogenation of carotene, it was applied to a comparative study of the hydrogenation ratios of a number of leaf carotenes. To insure the complete hydrogenation of these carotenes a large weight ratio of catalyst to carotene was employed. The weight of catalyst was always more than 5 times the weight of carotene. A summary of results in Table IV shows that the hydrogenation ratios for all of these leaf carotenes is nearly 10, but somewhat lower than that for carotene from carrot roots.

The hydrogenation ratio of lycopin was also determined and found to be 13 (*cf.* Table IV, Experiment 11, and Table V, Experiments 6 and 7). This value agrees with that reported by Karrer and Widmer (5) and indicates that the two samples of lycopin had the same degree of unsaturation.

Hydrogenations were also carried out in the mixed solvent, di-*n*-butyl ether and glacial acetic acid. In common with *p*-menthane and acetic acid, this solvent had a low vapor pressure; in contrast it was homogeneous. A comparison of the results set forth in Tables III and IV with those in Table V shows that within the experimental error the same values were obtained in the two solvents.

Dihydrocarotene was prepared in order to determine its hydro-



TABLE IV  
*Solvent, 3 Cc. Glacial Acetic Acid and 3 Cc. p-Menthane*

Experiment No.	Source of carotene	M.p. corrected °C.	Composition		Substance mg.	Catalyst mg.	Baro- metric pressure mm.	Temper- ature °C.	H <sub>2</sub> absorbed		Hydro- genation ratio
			C	H					cc.	mols × 10 <sup>4</sup>	
1	Fig leaves	175.2-175.7	89.29	10.61	2.697	5.027	17 661	768.2	1.165	48.30	9.61
2*	Sugar-beet leaves	180.2-180.8	89.43	10.49	2.988	5.570	18 406	765.7	1.355	55.87	10.03
3	Chard	180.2-180.8	89.56	10.50	2.791	5.202	20 767	764.5	1.215	50.48	9.70
4	Spinach	179.1-180.1	89.17	10.65	3.566	6.647	20 883	755.0	1.575	65.51	9.85
5	"	179.1-180.1	89.17	10.65	3.202	5.968	21.611	766.5	1.390	58.45	9.79
6	Alfalfa	179.7-180.2	89.43	10.53	2.645	4.930	17 329	756.8	1.170	48.66	9.87
7	"	179.7-180.2	89.43	10.53	3.419	6.373	21.130	755.6	1.525	63.31	9.93
8†	Cauliflower	181.1	89.39	10.44	3.112	5.801	22 206	762.4	1.345	56.03	9.66
9	Sunflower	176.6-178.5	89.31	10.59	2.941	5.482	17 917	761.7	1.270	52.06	9.50
10	Carrot roots	172.7-174.2	89.54	10.47	2.727	5.083	17 214	757.5	1.245	51.37	10.10
11‡	Lycopin, tomato fruit	166.8-167.9	89.07	10.34	2.201	4.103	21 151	757.4	1.290	52.53	12.80

\* Sugar-beet carotene was treated with fullers' earth before recrystallization.

† The solvent was given a presaturation with 20.415 mg. of catalyst before adding the reaction catalyst and sample tube.

‡ The duplicate analysis gave a lower result and contained ash; C, 88.45; H, 10.26; ash, 1.62. The ash was probably glass which was sucked in when the evacuated ampoule was opened.

TABLE V  
*Solvent, 5 Cc. of Di-n-Butyl Ether-Glacial Acetic Acid Mixture*

Experi- ment No.	Source of substance	M.p. corrected °C.	Composition		Substance		Catalyst	Baro- metric pressure mm.	Tem- pera- ture °C.	H <sub>2</sub> absorbed		Hydro- genation ratio
			C	H	mg.	mols × 10 <sup>6</sup>				cc.	mols × 10 <sup>6</sup>	
1	Stilbene	124.0-124.5	93.26	6.67	2.621	14.55	21.513	765.3	22.0	2.465	102.45	7.041
2	Carotene, sugar-beet leaves	180.2-180.8	89.43	10.49	2.396	4.468	22.552	762.7	25.8	1.080	44.17	9.89
3	" spinach	179.1-180.1	89.17	10.65	2.865	5.340	26.446	765.0	21.7	1.270	52.81	9.89
4	" sunflower	176.6-178.5	89.31	10.59	3.080	5.741	24.134	762.3	26.2	1.360	55.51	9.67
5	Dihydrocarotene, carrot roots		89.09	10.97	3.097	5.751	20.990	759.7	22.2	1.220	50.30	8.75
6	Lycopin, tomato fruit	167.9-169.0	89.29	10.22	1.965	3.662	14.185	760.3	25.3	1.190	48.59	13.27
7	Lycopin,* "	166.8-167.9	89.07	10.34	2.900	5.405	26.072	755.7	24.5	1.765	71.82	13.29

\* The duplicate analysis gave a lower result and contained ash; C, 88.45; H, 10.26; ash, 1.62. The ash was probably glass which was sucked in when the evacuated ampoule was opened.

generation ratio. For this purpose a sample of carotene was prepared which was divided into two portions. One portion was analyzed and catalytically hydrogenated (*cf.* Table IV, Experiment 10); the other was reduced with aluminum amalgam, then analyzed and hydrogenated catalytically (*cf.* Table V, Experiment 5). The analytical results showed that the aluminum amalgam treatment added only 1 mol of hydrogen and did not polymerize the molecule. The hydrogenation data showed that the hydrogenation ratio had been lowered from 10 to 9.

The discrepancy between these results and those of Karrer and Morf (4) who found that dihydrocarotene absorbed 10 mols of hydrogen on catalytic hydrogenation may mean that there is a fundamental difference between the carotenes employed.

#### CONCLUSION

From the results obtained it may be concluded: that all the leaf carotenes examined possess the same hydrogenation ratio, that under the conditions employed these carotenes absorb 10 mols of hydrogen per formula weight of pigment, and that they have only ten double bonds in the molecule; that dihydrocarotene, prepared by the reduction of carotene with aluminum amalgam, possesses a hydrogenation ratio of 9, corresponding to one double bond less than carotene; that lycopin, which has been obtained from California tomatoes, has thirteen double bonds and so necessarily has an open chain structure.

#### SUMMARY

1. A micro hydrogenation method has been developed which gives results accurate to approximately 2 per cent.
2. The hydrogenation ratios of carotenes from several sources have been obtained. These carotenes absorb close to 10 mols of hydrogen per formula weight of pigment.
3. Carotene reduced by aluminum amalgam has been found to lose only one double bond.
4. Lycopin has been shown to have a hydrogenation ratio of 13 in agreement with the results of other investigators.

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# DIRECTIVE INFLUENCES IN BIOLOGICAL SYSTEMS

## I. SPECIFICITIES OF LIPASE ACTIONS

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### INTRODUCTION

The chemical study of biological systems, or the chemistry of the changes occurring in living matter, is of such apparent complexity that any one who, at the present time, seriously attempts to carry on an experimental investigation in this field may possibly be likened to one who rushes in "where angels fear to tread." However, an honest realization of the difficulties of the problems and of the limitations of the methods of study will minimize the dangers of such an investigation.

The nature of the problem was presented clearly by Levene (1) in his address "The revolt of the biochemists" where it was shown that in the chemical behavior of certain biological systems a number of factors or substances are involved, and that the evaluation of these factors is not only possible but is actively proceeding.

The view is evident, and has been emphasized repeatedly (2) that enzymes and enzyme actions are important, and perhaps essential, directive influences in life processes. An attempt to determine experimentally certain of the factors which bear upon the directive influences of enzyme actions will be presented in this paper.

The enzyme work immediately preceding the present investigation may be outlined briefly. This work included studies of tumors and normal tissues of animal and human origin and was presented in a series of papers published in 1921 to 1928.<sup>1</sup>

In the main, the ester-hydrolyzing actions were studied. These

<sup>1</sup> These papers were published in *The Journal of Biological Chemistry*, *The Journal of General Physiology*, *The Journal of Cancer Research*, and *The Journal of the American Chemical Society*.

were chosen because of (1) the definite compositions of the substrates (ten simple esters were used as a rule), (2) the definite changes brought about by the enzymes and the simple method of their estimations, (3) the solubility of the substrates, (4) the fact that the enzyme materials acted upon these substrates to different extents. The conclusions of these studies included in brief the differences in the relative and absolute actions of the different tissues of an animal and of different animals, the influence of the age of the animal upon the actions and the finding of an "embryo" type of action, the actions of various tumors of animal and of human origin, and the similarity in the relative actions of certain tumor types to the embryo type.

The problem to be studied here may be outlined as follows: If to a small quantity of an active lipase material different inactive proteins or active tissue preparations are added, the actions may be greatly increased or modified. These changes may be due in part to the stabilizing action of the added colloidal protein. If the actions of a number of esters are due to a complex enzyme molecule in the original material, then the various added proteins (for example) would be expected to modify the actions on the different esters to very much the same extent. If, however, the actions are due to one definite, perhaps simple, enzyme grouping, then different proteins might show different specific actions with the different esters, and the actions might be modified to different extents. In the actual carrying out of such an experiment, conditions may not be so clear cut and simple in any given case. The original active enzyme might contain various substances which in themselves may modify or control the actions. However, it will be shown that with a proper choice of materials, definite conclusions can be drawn from the results.

#### EXPERIMENTAL

A large mass of experimental data has been accumulated. In place of giving this material in detail, individual experiments or averages of several experiments will be presented as representative of the general run of results. The actions of active pancreas lipase in the presence of proteins, inactive in themselves, and of a number of lipolytically active normal tissue and tumor extracts will be given.

A commercial pig pancreas material, prepared by extraction of the minced gland with ether and acetone, and obtainable in quantity with constant properties, was used in many of the experiments. The material contained 10 per cent sodium chloride, but this did not interfere with the study, as a pancreas material prepared similarly in the laboratory but without the added sodium chloride gave essentially the same results.

The actions in the presence of proteins will first be given. The results will be limited to a few concentrations of the lipase material

TABLE I

*Ester-Hydrolyzing Actions in Terms of Tenths of Milli-Equivalents of Acid Produced by Lipase Material and Casein*

	Lipase material solution				Casein solution, 17.8 mg. per cc.
	0.22 mg. per cc.			0.11 mg. per cc., 22 hrs., 37°, then tested	
	Immediate testing	22 hrs., 20°, then tested	22 hrs., 37°, then tested		
PhOAc. ....	0 86	0 68	0 07	0 02	0 51
Gl(OAc) <sub>3</sub> .....	1 30	0 70	0 37	0 31	0 49
MeOCOPr. ....	1 08	0 76	0 32	0 22	0 05
PhCH <sub>2</sub> OAc.....	1 08	0 62	0 29	0 24	0 02
EtOAc. ....	0 20	0 13	0 07	0 03	0 18
MeOAc. ....	0 23	0 14	0 08	0 04	0 37
EtOCOPr.....	0 71	0 49	0 21	0 12	0 02
MeOBz. ....	0 00	0 00	0 00	0 00	0 04
EtOBz. ....	0 00	0 00	0 00	0 00	0 00
IsobuOAc.....	0 40	0 20	0 14	0 06	0 00

and of the proteins. The mixtures were prepared by dissolving 0.1 gm. (or in a few cases 0.05 and 0.2 gm.) of the former in 300 cc. of water or protein solution containing either 0.4 or 0.8 gm. of the protein. After different periods of time these solutions were diluted with water to 450 cc. and duplicate 15 cc. portions used for the tests. The concentrations of the mixtures tested therefore corresponded to 0.22 mg. (0.11 or 0.44 mg. in a few cases) of enzyme material per cc. and 8.9 or 17.8 mg. of protein per cc. Toluene was present throughout. Unless otherwise stated, the mixtures initially were brought to pH 7.0. The ten esters in the same



quantities as in the earlier work were used, as follows (very close to 3.4 milli-equivalents of each): 0.43 cc. of phenyl acetate, 0.21 cc. of glyceryl triacetate, 0.39 cc. of methyl butyrate, 0.49 cc. of benzyl acetate, 0.33 cc. of ethyl acetate, 0.27 cc. of methyl acetate, 0.44 cc. of ethyl butyrate, 0.42 cc. of methyl benzoate, 0.49 cc. of ethyl benzoate, and 0.44 cc. of isobutyl acetate. The esters are listed in the same arbitrary order as in the former papers. The ester-hydrolyzing actions were measured by titration with 0.1 N sodium hydroxide solution with phenolphthalein as indicator and are given as cc. of 0.1 N alkali used, corrected for ester blanks, or tenths of milli-equivalents of esters hydrolyzed. The actions are given for 22 hours at 37°.

In Table I are shown the lipase material actions on the esters without any added proteins. The actions of the casein solution alone (prepared with purified casein) are also shown. These last are appreciable for a few esters and may be due to the special preparation used. The actions of other proteins on the esters alone were practically zero. The water-ester blanks were subtracted from the actions in every case. The lipase material when tested immediately after the solutions were made up gave actions of considerable magnitude, but if the solutions were allowed to stand for 22 hours at 37° before the addition of the esters, the actions were quite small.

Table II contains the results of the action of the lipase material upon the esters in the presence of proteins, corrected for ester-water blanks only. Columns 1 to 3 were obtained under similar experimental conditions: mixing of 300 cc. of the protein solution and lipase material at pH 7.0, allowing the solutions to stand at 37° for 24 hours, diluting with water to 450 cc., verifying and correcting the pH, setting up duplicate 15 cc. portions with each of the esters and necessary blanks, incubating for 22 hours at 37°, and titrating the acid formed.

It is evident that the action on phenyl acetate in the presence of albumin was much greater than in the presence of the other proteins, while with casein the action on benzyl acetate was much smaller. The remaining actions did not differ to any great extent for the different esters in the presence of the three proteins. Columns 4 to 7 in Table II show the change in actions if the lipase material was allowed to stand in the gelatin solution for different

lengths of time before the addition of the esters. The action was decreased considerably for phenyl acetate, less for glyceryl tri-

TABLE II  
*Ester-Hydrolyzing Actions in Terms of Tenths of Milli-Equivalents of  
Acid Produced by Lipase Material-Protein Mixtures*

Lipase material, 0.22 mg. per cc.

	17.8 mg. per cc., 24 hrs., 37°, then tested			Gelatin, 8.9 mg. per cc.			
	Gelatin	Casein	Albu- min	Imme- diate testing	37°, 2 hrs., then tested	37°, 6 hrs., then tested	37°, 24 hrs., then tested
	(1)	(2)	(3)	(4)	(5)	(6)	(7)
PhOAc . . . . .	0 76	0 96	2 03	1.45	1.49	1.20	0.61
Gl(OAc) <sub>3</sub> . . . . .	2.60	2.69	2 63	2.77	2 85	2.47	2.05
MeOCOPr . . . . .	1.92	1.81	2.11	2.07	2.18	2.05	1.62
PhCH <sub>2</sub> OAc . . . . .	1.98	1.15	2.38	1.93	2.07	1.89	1.74
EtOAc . . . . .	0.48	0.41	0.36	0 37	0.41	0.43	0.42
MeOAc . . . . .	0 48	0.57	0 38	0.35	0.45	0.39	0.41
EtOCOPr . . . . .	1.08	1.00	1 12	1.11	1.24	1.18	1.02
MeOBz . . . . .	0 03	0.10	0.03	0.05	0.11	0.07	0.03
EtOBz . . . . .	0 04	0.12	0.04	0.06	0.02	0.06	0.00
IsobuOAc . . . . .	0 86	0.58	0.66	0.81	0.88	0.88	0.80

	8.9 mg. per cc.				8.9 mg. per cc., 24 hrs., 37° at pH 5.0, then tested at pH 7.0	
	37°, 24 hrs., then tested. Albumin	Immediate testing			Gelatin	Albumin
	(8)	Edestin (9)	Ghadin (10)	Corn gluten (11)	(12)	(13)
PhOAc . . . . .	1 67	0 95	0 35	0.78	0.62	1.23
Gl(OAc) <sub>3</sub> . . . . .	2.20	2 09	0.42	0.75	2.13	1 80
MeOCOPr . . . . .	1.72	1 56	0.42	0.94	1.52	1.28
PhCH <sub>2</sub> OAc . . . . .	1.79	1.63	0.56	0.68	1.60	1.46
EtOAc . . . . .	0.27	0.33	0 02	0.22	0.36	0.23
MeOAc . . . . .	0.33	0 33	0.06	0.23	0.32	0.25
EtOCOPr . . . . .	1 03	1.01	0.27	0.70	0.96	0.78
MeOBz . . . . .	0.02	0 02	0.06	0.10	0.04	0.00
EtOBz . . . . .	0.00	0.04	0.05	0.06	0.04	0.00
IsobuOAc . . . . .	0.63	0.67	0 15	0.36	0.72	0.45

acetate and methyl butyrate, and practically not at all for the other esters. Columns 8 to 11 (Table II) show first that the effect of half the amount of albumin was to decrease the ester-

hydrolyzing actions as compared with the results in Column 4 but only to small extents, not proportionately, and then the actions in the presence of three additional proteins. The behavior of edestin was similar to that of casein (except for benzyl acetate). Gliadin decreased markedly the actions in comparison with the actions of the lipase material alone, while corn gluten showed little or no effect.

Columns 12 and 13 show the manner in which the relative effects of different proteins may be emphasized by small changes in the experimental conditions. Upon allowing the protein-lipase material mixtures to stand at 37° for 24 hours at pH 5.0 (instead of pH 7.0), then bringing them to pH 7.0, diluting, and adding the esters, twice as much action was found on phenyl acetate in the presence of albumin as in the presence of gelatin, but distinctly less on all the other esters.

The striking features of these results are the actions on phenyl acetate and to a less extent on benzyl acetate in comparison to the actions on the other esters. If the protein action was solely stabilizing or protective, no such differences would be expected. In the former papers on enzyme actions it was pointed out in various connections that as many substrates as possible should be used to bring out possible differences in the properties of the enzyme materials. This is illustrated clearly in the present instance. If phenyl acetate had not been used, the actions of the proteins might have been interpreted as merely protective. With phenyl acetate, the picture is different, and while protective action unquestionably is exerted, there are other actions due to the specific proteins, and presumably modifying or influencing the action of a lipase or ester-hydrolyzing grouping of the lipase material. The use of a larger number of proteins, or of a greater variety of esters, might bring out such relations even more strikingly.

In order to study the possible actions of protective colloids alone, experiments were carried out with the lipase material and various concentrations of silica gel, kaolin, alumina cream, and infusorial earth. No protective action was evident when the lipase material was allowed to stand with any of these materials before testing with the esters. These results will not be given in detail.

The behavior of the lipase material in the presence of extracts of

beef kidney, beef lung, and beef liver will next be presented. The actions on the esters in each case, for example lipase material and beef kidney, may be considered in several different ways. The actions of a given mixture of lipase material and beef kidney are made up of the separate actions of the two plus the accelerating or retarding actions exerted by each material upon the other. There is no conclusive method of presenting the results so as to show such possible influences in an entirely satisfactory manner. The simplest method which will be used and which will bring out

TABLE III

*Ester-Hydrolyzing Actions in Terms of Tenths of Milli-Equivalents of Acid Produced by Beef Kidney Extracts Alone and with Lipase Material*

Beef kidney extract 17.8 mg. per cc.; lipase material 0.22 mg. per cc.

	Immediate tests			Column 3 less Columns 1 + 2	Incubated 24 hrs., 37°, then tested			Column 7 less Columns 5 + 6
	Kid- ney	Lipase mate- rial	Kidney + lipase mate- rial		Kid- ney	Lipase mate- rial	Kidney + lipase mate- rial	
	(1)	(2)	(3)		(5)	(6)	(7)	
PhOAc .....	3.02	0.86	3.21	-0.67	2.79	0.07	2.85	-0.01
Gl(OAc) <sub>3</sub> .....	3.42	1.30	3.86	-0.86	3.30	0.37	3.80	0.13
MeOCOPr .....	1.18	1.08	2.49	0.23	0.97	0.32	2.28	0.99
PhCH <sub>2</sub> OAc .....	1.88	1.08	2.41	-0.55	1.92	0.29	2.36	0.15
EtOAc .....	1.38	0.20	1.47	-0.11	1.33	0.07	1.38	-0.02
MeOAc .....	1.27	0.23	1.38	-0.12	1.19	0.08	1.25	-0.02
EtOCOPr .....	1.11	0.71	2.05	0.23	0.96	0.21	1.59	0.42
MeOBz .....	0.16	0.00	0.23	0.07	0.15	0.00	0.17	0.02
EtOBz .....	0.11	0.00	0.17	0.06	0.11	0.00	0.12	0.01
IsobuOAc .....	1.66	0.40	1.87	-0.19	1.67	0.14	1.75	-0.06

certain relations, consists in giving the actions of lipase material alone, of beef tissue extract alone, of the mixture of the two, and finally the latter minus the sum of the first two. This should indicate whether the lipase material and the beef tissue exerted inhibiting or accelerating actions on each other or had no effect.

In Table III are given typical results obtained with beef kidney extract and lipase material. Two sets of results are shown; one in which the esters were added immediately after the kidney and

lipase material were mixed, the other in which the kidney-lipase material mixture was incubated for 24 hours at 37° before the esters were added.

The actual actions in the two series with different times of standing (Columns 3 and 7) did not differ much for practically all the esters, ethyl butyrate showing the greatest difference. With the corrected actions (Columns 4 and 8) one fact stands out. The actions on the esters were influenced differently; in other words,

TABLE IV  
*Ester-Hydrolyzing Actions in Terms of Tenths of Milli-Equivalents of Acid  
Produced by Beef Lung Extracts Alone and with Lipase Material*  
Beef lung extract 17.8 mg. per cc.

	Lipase material 0.22 mg. per cc.						Incubated 24 hrs., 37°, then tested. Lipase material per cc.		Column 8 less Column 7
	Immediate tests		Column 2 less correc- tions	Incubated 24 hrs., 37°, then tested		Column 5 less correc- tions			
	Lung	Lung + lipase mate- rial		Lung	Lung + lipase mate- rial				
	(1)	(2)		(3)	(4)		(5)	(6)	
PhOAc.....	4 11	4.76	-0.21	3.53	3 53	-0 07	3.03	3 28	0 25
Gl(OAc) <sub>3</sub> .....	1.32	2 56	-0.06	1.29	2 84	1 18	2.06	3.47	1 41
MeOCOPr.....	2.83	3.33	-0.58	2.72	3 24	0 20	2 85	3.63	0.78
PhCH <sub>2</sub> OAc. ....	0 91	1.98	-0.01	0.96	2.24	0 99	1.68	2.76	1.08
EtOAc.....	0 90	1.09	-0 01	0 92	1.13	0.14	1 17	1 46	0 29
MeOAc.....	1.39	1.43	-0.20	1 42	1.43	-0.07	1.61	1 85	0.24
EtOCOPr.....	1 61	2 21	-0 21	1.53	2 04	0.30	1.92	2 76	0.84
MeOBz.....	0.47	0.53	0.06	0.43	0.47	0.04	0.45	0 58	0.13
EtOBz.....	0.34	0.41	0.07	0.34	0 37	0.03	0.33	0.47	0.14
IsobuOAc..	0.79	1.27	0.08	0.83	1.41	0.44	1.41	1.95	0.54

selective or specific effects were observed. This was true, no matter which method of applying the corrections was used. The actions on methyl and ethyl butyrates were either greatly increased or practically unchanged, and on phenyl acetate and glyceryl triacetate unchanged where the former were increased, and greatly decreased where the former remained constant. The remaining esters occupied intermediate positions.

In considering the corrected actions, the writer considers Columns 4 and 8 to give a reasonable interpretation of the results

and to present true relations. Both columns lead to the same conclusions, but the results in Column 8 are especially striking.

The results in Table IV refer similarly to beef lung extract and lipase material mixtures. The lipase material actions alone were the same as those given in Table III and are therefore omitted. The corrected actions given in Columns 3 and 6 again showed specific influences, but different from those shown in Table III. The results of Column 6 can again be most readily interpreted. Greatly increased actions were obtained on glyceryl triacetate and

TABLE V

*Ester-Hydrolyzing Actions in Terms of Tenths of Milli-Equivalents of Acid Produced by Beef Liver Extracts Alone and with Lipase Material*

Beef liver extract.....	17.8 mg. per cc.		4.5 mg. per cc.			
Lipase material.....	0.22 mg. per cc.		0.22 mg. per cc.			
	Immediate tests		Incubated 24 hrs., 37°, then tested			
	Liver (1)	Liver + lipase material (2)	Liver (3)	Liver + lipase material (4)	Liver (5)	Liver + lipase material (6)
PhOAc .....	6 42	6.29	7.45	7.07	4.31	2 52
Gl(OAc) <sub>3</sub> .....	5.10	5.27	6 84	6.79	4.06	1.94
MeOCOPr .....	5 72	5.91	6.46	6 60	3.90	2 78
PhCH <sub>2</sub> OAc .....	3.14	3.21	4.08	3.63	2 31	1.44
EtOAc .....	3 70	3.76	4.47	4.42	2.14	1.27
MeOAc. . . . .	4.39	4.24	5.05	4.84	2.55	1.77
EtOCOPr...	4 06	4.01	4.30	4.68	2.12	1.87
MeOBz. . . . .	0.99	0.85	1.06	1.10	0.40	0.54
EtOBz. ....	0.48	0 76	0.79	0.77	0.27	0.30
IsobuOAc....	3 45	3.50	3 98	3.94	2.17	1.49

benzyl acetate, somewhat less on isobutyl acetate, still less on ethyl and methyl butyrates, and negligible influences on the remaining esters.

Columns 7 and 8 (Table IV) showed the uncorrected results obtained with the beef lung extract and different concentrations of lipase material, while in Column 9 are given the differences between the actions in Columns 7 and 8. These last values are caused by the increased amounts of lipase material and are independent of corrections for lung and lipase material blanks. Spe-

cific increases are apparent, greatest on glyceryl triacetate and benzyl acetate, next on methyl butyrate and ethyl butyrate, next on isobutyl acetate, and small or negligible on the rest. The larger amounts of lipase material apparently increased the influence on the butyrates to a greater extent than on the other esters;

TABLE VI

*Ester-Hydrolyzing Actions in Terms of Tenths of Milli-Equivalents of Acid Produced by Human Type I Fibroid Extracts Alone and with Lipase Material*

Type I Fibroid Extracts R105A and R109A, 44.4 mg. per cc.; lipase material, 0.11 and 0.22 mg. per cc.; incubated 24 hours at 37°, and esters then added and tested.

	Fibroid Extract R105A			Fibroid Extract R109A			Actions corrected for fibroid extracts and lipase materials			
	Alone	Lipase material		Alone	Lipase material		Fibroid Extract R105A		Fibroid Extract R109A	
		0.11 mg. per cc.	0.22 mg. per cc.		0.11 mg. per cc.	0.22 mg. per cc.	Plus 0.11 mg. lipase material per cc.	Plus 0.22 mg. lipase material per cc.	Plus 0.11 mg. lipase material per cc.	Plus 0.22 mg. lipase material per cc.
PhOAc .....	0.97	1.15	1.07	0.68	0.74	0.68	0.16	0.03	0.04	-0.07
Gl(OAc) <sub>3</sub> .....	0.49	2.88	3.80	0.42	2.28	2.86	2.08	2.94	1.55	2.07
MeOCOPr .....	0.40	2.40	2.93	0.29	1.82	2.18	1.78	2.21	1.31	1.57
PhCH <sub>2</sub> OAc .....	0.23	2.28	2.85	0.12	1.51	2.01	1.81	2.33	1.15	1.60
EtOAc .....	0.19	0.57	0.78	0.12	0.42	0.69	0.35	0.52	0.27	0.50
MeOAc .....	0.26	0.55	0.83	0.16	0.46	0.65	0.25	0.49	0.26	0.41
EtOCOPr .....	0.19	1.22	1.86	0.14	1.16	1.51	0.91	1.46	0.90	1.16
MeOBz .....	0.16	0.27	0.13	0.04	0.06	0.00	0.11	-0.03	0.02	-0.04
EtOBz .....	0.02	0.06	0.12	0.04	0.00	0.00	0.04	0.10	-0.04	-0.04
IsobuOAc .....	0.23	0.94	1.45	0.12	0.86	1.12	0.65	1.08	0.68	0.86

but the predominating effects on glyceryl triacetate and benzyl acetate are evident in all the results presented in Table IV.

The results obtained with beef liver extracts and lipase material shown in Table V must be considered somewhat differently. Columns 1 to 4 present the actions obtained in the same manner as the beef kidney and beef lung extracts results; namely, with an extract corresponding to 17.8 mg. of beef liver extracted per cc. of final mixture. The ester-hydrolyzing actions of this extract alone

were quite large and the addition of lipase material did not influence them at all. With smaller concentrations (4.5 mg. per cc.) as shown in Columns 5 and 6, the lipase material decreased the actions on the esters. The greatest decreases were observed with phenyl acetate and glyceryl triacetate, the smallest with methyl and ethyl benzoates (slight increases as a matter of fact for these). The decreases were of quite considerable extent for most of the esters.

In order to make the presentation of this part of the problem more complete, some results obtained with the lipase material and extracts of human tumors will be presented.

It may be recalled that human uterine fibroids were divided into two groups on the basis of their lipase actions; Type I, small absolute actions, curves or relative actions similar to those found with certain malignant human tumors, several transplantable rat tumors, and the embryo type of action; and Type II, larger absolute actions, curves or relative actions similar to those found with certain benign tumors and human uterine muscle.

The results obtained with added lipase material and Type I fibroid extracts are presented in Table VI, and those for Type II fibroid extracts in Table VII.

With the Type I fibroids, the actions of the fibroid-lipase material mixtures were equal to the sums of the separate actions with phenyl acetate and methyl and ethyl benzoates. The actions were much greater for the mixtures with glyceryl triacetate, almost as great with methyl butyrate and benzyl acetate, somewhat less so for ethyl butyrate, followed by isobutyl acetate, while with methyl acetate and ethyl acetate the increased actions, while not very large, were marked and unquestionable.

With the Type II fibroids, the actions of the fibroid extracts alone were quite large for most of the esters. The actions of the mixtures as compared with the separate actions were not uniform for the different preparations. The increased actions were greatest for glyceryl triacetate, benzyl acetate, and isobutyl acetate, but it may be pointed out that the fibroid actions alone on these three esters were not large initially.

In comparing the Type I and Type II results, it is of interest to note that while the relative actions of the two types are completely distinctive and characteristic, when the lipase material was added



TABLE VII

*Ester-Hydrolyzing Actions in Terms of Tenths of Milli-Equivalents of Acid Produced by Human Type II Fibroid Extracts Alone and with Lipase Material*

Type II Fibroid Extracts R104A, R101A, and R112B, 44.4 mg. per cc.: lipase material, 0.11 and 0.22 mg. per cc.; incubated 24 hours at 37°, and esters then added and tested.

	Fibroid Extract R104A			Fibroid Extract R101A			Fibroid Extract R112B	
	Alone	Lipase material		Alone	Lipase material		Alone	Lipase material
		0.11 mg. per cc.	0.22 mg. per cc.		0.11 mg. per cc.	0.22 mg. per cc.		0.22 mg. per cc.
PhOAc.....	2.44	2.42	2.46	1.17	0.88	0.70	0.93	1.50
Gl(OAc) <sub>3</sub> .....	0.96	2.79	3.92	0.51	2.07	2.60	0.44	2.11
MeOCOPr.....	3.36	3.85	4.35	2.19	2.33	2.23	1.70	2.58
PhCH <sub>2</sub> OAc.....	0.53	2.23	3.16	0.22	1.73	2.05	0.08	1.50
EtOAc.....	1.26	1.44	1.62	0.74	0.74	0.69	0.61	0.97
MeOAc.....	1.33	1.52	1.68	0.80	0.82	0.74	0.72	1.02
EtOCOPr.....	2.90	3.21	3.55	1.95	1.90	1.57	1.53	2.25
MeOBz.....	1.05	1.15	1.09	0.52	0.46	0.28	0.41	0.51
EtOBz.....	0.86	0.98	0.90	0.45	0.35	0.21	0.36	0.43
IsobuOAc.....	0.56	1.05	1.78	0.23	0.84	1.04	0.13	0.94

Actions corrected for fibroid extracts and lipase material

	Fibroid Extract R104A		Fibroid Extract R101A		Fibroid Extract R112B
	Plus 0.11 mg. lipase material per cc.	Plus 0.22 mg. lipase material per cc.	Plus 0.11 mg. lipase material per cc.	Plus 0.22 mg. lipase material per cc.	Plus 0.22 mg. lipase material per cc.
PhOAc.....	-0.04	-0.05	-0.29	-0.54	0.50
Gl(OAc) <sub>3</sub> .....	1.52	2.59	1.25	1.72	1.30
MeOCOPr.....	0.27	0.67	-0.08	-0.28	0.56
PhCH <sub>2</sub> OAc.....	1.46	2.34	1.27	1.54	1.13
EtOAc.....	0.15	0.29	-0.03	-0.12	0.29
MeOAc.....	0.15	0.27	-0.02	-0.14	0.22
EtOCOPr.....	0.19	0.44	-0.17	-0.59	0.51
MeOBz.....	0.10	0.04	-0.06	-0.24	0.11
EtOBz.....	0.12	0.04	-0.10	-0.24	0.07
IsobuOAc.....	0.43	1.08	0.55	0.67	0.67

the actions were increased in such a way that the resulting actions could not be distinguished. For example Fibroid Extracts R109A (Type I) and R101A (Type II) while entirely different when tested directly were practically identical if 0.22 mg. of lipase material per cc. were present.

The results with uterine muscle extracts showed exactly the same relations and changes when lipase material was added as did the Type II fibroid extracts, and will therefore not be given in detail.

### *Summary of Experimental Results*

In summarizing the experimental work presented here, it may appear that the use of ten esters for which the results are given in this paper was unnecessary. With any two systems, it is true that the differences in actions might be limited to two or three esters, but there are reasons for presenting the results for the ten. In the first place, the esters which brought out differences were different even for the limited number of systems used, so that more than two or three esters would be necessary for general comparisons. Further, these ten esters were used in the general study of tumors and normal tissues and comparison with the results obtained with them would be possible when desired. In order to avoid at least some of the experimental difficulties inherent in colloidal systems, soluble esters only, and not fats, were used. Of the esters studied, glyceryl triacetate may be considered the closest approach to a fat, and the results with this ester are perhaps of more than ordinary interest.

In considering the experimental results, it must be kept in mind that the differences and regularities found obtain with the specific preparations used. For example, a different enzyme preparation might behave differently even if obtained from the same species of animal. A different gelatin might show somewhat different specific effects, etc. In fact, such variations have been observed but are not reported in detail here. The variations are, in a way, of secondary significance until such time as the compositions, structures, and properties of complex proteins, etc., are more definitely known. The results presented apply, strictly speaking, only to the systems used.

With lipase material and proteins lipolytically inactive in them-

selves the most striking differences were observed in the actions on phenyl acetate. In the presence of albumin, the actions were much (2 to 3 times) greater than in the presence of gelatin, casein, or edestin, while the actions on the other esters did not differ to any marked extent.

The lipase material added to the different beef tissue extracts showed marked differences. With beef kidney, actions were most favored on methyl and ethyl butyrates, while actions on phenyl acetate, glyceryl triacetate, and benzyl acetate were most inhibited. With beef lung, actions on glyceryl triacetate and benzyl acetate were most favored. With beef liver, the more concentrated extracts showed no differences in actions with added lipase material, while with the more dilute extracts, decreased actions on all the esters were observed.

The interesting feature in adding the lipase material to the Type I and Type II human uterine fibroid extracts was that while the two types alone showed entirely distinctive actions, in the presence of added lipase material the actions were practically identical. The increases with the Type I fibroids were largest on glyceryl triacetate, benzyl acetate, the butyrates, and isobutyl acetate; with the Type II fibroids they were largest on glyceryl triacetate and benzyl acetate, next on isobutyl acetate, and small on the remaining esters.

The question of correcting the actions for blanks (separate actions of lipase material and tissue extracts) while important, may be set aside for the moment in the consideration of the results. The same method of correction must be applied to all the different mixtures and the comparative actions and changes with these mixtures will remain the same regardless of the corrections and the method of applying them.

Another point must be emphasized. The results are given for one definite concentration of ester in each case, and for a limited number of concentrations of lipase material, protein, and tissue extracts. It would evidently be possible and, in fact, not difficult, to vary the amounts of the esters, as well as of the lipase material, extracts, and conditions of testing, to bring out differences and similarities in a clearer and more striking manner. The results with gelatin and albumin shown in Columns 13 and 14 of Table II may serve to indicate the possibilities in a simple way. An

extended study of this nature would be of considerable interest but was not carried out mainly because it was desired to develop general principles, if possible, rather than complete experimental studies of the relations.

Formol titrations, carried out in every experiment in which lipase material was used, gave evidence of small amounts of protease action on casein and edestin, but in no other case. Whether protease action was responsible for the decrease in the liver lipase cannot be definitely stated, since the formol titration values were zero with them. As a matter of record, it may be stated that the lipase material contained active amylase.

#### DISCUSSION

The primary reason for the choice of lipase actions in the study of directive influences in biological systems was dictated by the fact that the substrates are definite in composition, and when acted upon undergo definite chemical changes. Simple esters were used and complicating factors such as optical isomerism, etc., were eliminated. The conditions throughout were kept as simple as possible, but this attempt at simplicity may have introduced factors which need further study and interpretation. For example, a highly active enzyme preparation was studied in the presence of certain proteins and tissue and tumor extracts. No other substances were added. The hydrogen ion concentrations of the mixtures changed as actions on the substrates proceeded, but it was considered that such changes were preferable for the theoretical considerations to adding buffer mixtures or possibly other substances to keep the hydrogen ion concentrations constant throughout the course of the actions. This view was adopted because it has been found by the writer, as well as by many other workers, that lipase actions are readily influenced by added substances.

The question raised in the "Introduction" may now be answered. The evidence which has been presented is best interpreted in the sense that the enzyme responsible for ester-hydrolyzing or lipase actions possesses a definite composition or is present as a definite grouping. The different actions observed when proteins or tissue extracts are present or added are due to their compositions and properties. Their actions cannot be considered solely or even essentially protective because of the striking specificities observed,

although a certain amount of stabilizing or protective action is certainly present. If the lipase material is present in a fairly concentrated form (it is manifestly meaningless at present to speak of purity in this connection), the added proteins or other substances might be expected to show very marked effects. If large amounts of proteins, etc., are present initially, the resulting changes would be smaller or not evident at all. The fact that tissue and tumor extracts in themselves frequently contain sufficient protein and other substances to mask any specific actions of other proteins, etc., on their own ester-hydrolyzing actions was shown in an earlier paper (3) in connection with the actions of mixtures of tumor and normal tissue extracts. The curves of the actions of these mixtures in all cases studied were made up additively of the curves of the separate extracts, although the absolute actions were somewhat less.

The following general principle may be stated:

If Enzyme E represents the concentrated or refined enzyme exerting small but definite actions on Substrates A and B, then when Protein P (or other activating substance) is added to Enzyme E, the mixture exerts greatly increased action on Substrate A but not on Substrate B; if Protein Q is added to Enzyme E, greatly increased action is shown on Substrate B but not on Substrate A. Also, Enzyme E may be an essential constituent of the protein not separable from it with the experimental means available at present.

	Actions on	
	Substrate A	Substrate B
Enzyme E. . . . .	+	+
Proteins P and Q. . . . .	0	0
E + P. . . . .	+++	+
E + Q. . . . .	+	+++
(PE). . . . .	+++	+
(QE). . . . .	+	+++

This general principle represents and correlates definite experimental facts. It does not attempt to explain them or to present a mechanism by which the action may be supposed to occur. At the same time, the view that lipase or ester-hydrolyzing actions are due to a definite grouping and that specificities of actions on

~~different~~ esters are due to other components of the molecule or ~~constituents~~ of the medium falls into line with work published a number of years ago (4), in which indirect evidence of the nature of such a grouping was presented.

Some of the work which has appeared in the literature and bearing directly upon this problem may be reviewed briefly. Various substances and preparations which increase the actions of enzymes have been called "coenzymes." This term does not add to the knowledge of the phenomena, and it has therefore seemed preferable to the writer to avoid it in the present connection. Much of the work in this field is not directly connected with the problem studied in this paper, although the results in many cases may be included in the general formulation.

In an extended study of the enzymes of the castor-bean a number of years ago, it was shown that water extracted an enzyme hydrolyzing ethyl butyrate more rapidly than glyceryl triacetate under certain definite conditions and that 10 per cent sodium chloride solution extracted an enzyme which hydrolyzed glyceryl triacetate to a greater extent than ethyl butyrate (4, 5). Willstätter and Waldschmidt-Leitz (6) stated that the lipase of castor-beans was completely insoluble in water and in sodium chloride solution and that the actions described were due to the proteolytic actions of the castor-bean. A reinvestigation of this subject (7) showed the incorrectness of Willstätter and Waldschmidt-Leitz's criticisms and confirmed and extended the work previously reported.

A different point of view, and one which is more nearly related to that presented in this paper was brought forward by Platt and Dawson (8). They showed that a pancreatic extract having definite hydrolytic actions on ethyl butyrate and on olive oil, had its actions modified by the addition of albumin which increased the first action and decreased the second, and by the addition of edestin (a globulin) which increased both, the second to a greater extent than the first. They concluded that the ester-hydrolyzing property of the enzyme had been definitely altered by changing the accompanying protein. They compared the actions of the pancreatic extract in the presence of albumin and edestin with the actions of the water extract (containing albumin) and sodium chloride solution extract (containing globulin) of the castor-bean.

Experiments (7) suggested by these results showed that further additions of albumin and edestin did not modify the actions of the castor-bean extracts. Here, evidently, the protein and other substances of the castor-bean were present in sufficient concentration to mask the effects of added proteins. The results of Platt and Dawson fall into line with the results presented here in more extended form.

Various problems related to the work described here might be discussed but would lead too far. It will be obvious that with the schematic representation of the general principle given on p. 68, actions of widely different types may be fitted into the scheme. It need hardly be necessary to state that Proteins *P* and *Q* should be taken as symbolic. They may represent a wide variety of substances.

It is apparent that in discussing enzyme actions (and the same may be said of chemical actions in general) the system as a whole must be considered. Taking one constituent and determining its action or reaction may give an incomplete and misleading picture (9) of the changes which occur in the complete system. At the present time, the various actions and interactions of the constituents of biological systems are not sufficiently well defined to make possible the prediction of the behavior of the whole from a knowledge of its parts. Such prediction is still uncertain even for inorganic systems except those of the simplest nature. It is necessary to study the system as a whole in addition to the study of its constituent parts.

To return once more to the schematic representation given on p. 68, an analogy, possibly far fetched, may be suggested. *E* may be considered to be the heredity factor, but the properties which appear in any given case depend upon the environment *P* or *Q*. *E* possesses any number of potential actions, but the ones which will develop depend upon the external factors. The properties of the system will depend upon *E* and *P* or *Q* and the reciprocal influences these may exert upon each other.

#### SUMMARY AND CONCLUSIONS

The hydrolyzing actions on ten different esters of mixtures of a pancreas preparation with a number of different proteins, beef tissue extracts, and human tumor extracts were studied.

Specific increases on various esters in comparison with the individual materials showed that the influences of the added materials on the pancreas preparation were not solely protective, but that interactions occurred which increased certain of the actions selectively.

It was concluded that the lipase or ester-hydrolyzing actions described here and in a number of earlier papers were due in all probability to a definite chemical grouping, and that the specificities found for tumors and normal tissues and for the mixtures described here were due to the protein and other substances characteristic of the material in question. These characteristic actions might be ascribed either to the addition or presence of such substances influencing a concentrated or refined enzyme preparation, or to a complex substance (molecule) containing the active enzyme grouping.

A schematic representation or formulation of this view was presented.

The importance in biological actions of considering the system as a whole was emphasized.

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# THE SITE OF THE SYNTHESIS OF HIPPURIC ACID AND PHENYLACETURIC ACID IN THE DOG\*

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At present the view is commonly accepted that hippuric acid is produced chiefly by the kidney in most animals and exclusively by that organ in the dog. This conclusion is based largely on the well known work of Bunge and Schmiedeberg (1) who were able to demonstrate that hippuric acid was formed when blood containing benzoic acid and glycine was perfused through dog kidney. Furthermore, they failed to find hippuric acid after injecting sodium benzoate and glycine into dogs in which the renal blood vessels were tied, but could find it when the ureters were ligated and the vascular supply of the kidneys left intact. Although these findings appear to constitute almost conclusive proof that in the dog, the synthesis of hippuric acid is effected only by the kidney, Kingsbury and Bell (2) challenged these conclusions. They reported that after injecting sodium benzoate and glycine into a nephrectomized dog, they were able to find hippuric acid in the blood and other tissues. Unfortunately, they did not differentiate between combined benzoic acid and hippuric acid, which is a serious error inasmuch as in the dog the conjugation with glycuronic acid is the more prominent. In only one experiment did they record the actual isolation of hippuric acid without, however, giving any information as to the amount. Snapper, Grünbaum, and Neuberg (3) criticized their conclusions and proceeded to reinvestigate the problem. They corroborated the work of Bunge and

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Schmiedeberg, Kochs (4), and Bashford and Cramer (5), finding that dog kidney on perfusion with sodium benzoate and glycine yielded hippuric acid. They further found that the same held true for the kidney of the human, of the pig, and to a limited extent of the sheep. They failed to find even a trace of hippuric acid in the tissues of nephrectomized dogs after injecting large doses of sodium benzoate and glycine. They concluded that, contrary to Kingsbury and Bell, they were able to establish the second conclusion of Bunge and Schmiedeberg: in the dog, hippuric acid is formed *only* in the kidney and in no other organ.

In species other than the dog, there is good evidence that the synthesis of hippuric acid is not confined solely to the kidney. Kühne and Hallwachs (6), in 1857, voiced the opinion that the synthesis took place within the vessels of the liver and in the presence of bile constituents. The reliability of their experimental work is subject, however, to grave doubt, and even in 1866 their conclusions were questioned by Meissner and Shepard (7) who considered the kidney as the more likely site for the synthesis. Their experimental results are, however, somewhat conflicting. They found, following the feeding of sodium benzoate, hippuric acid in the blood of a rabbit in which the renal blood vessels were ligated, but they also found it in the blood of rabbits and cats in which the portal vein had been tied. These observations are somewhat comparable to those made by Bunge and Schmiedeberg (1) on the frog. In this animal neither the removal of the kidney nor of the liver apparently stopped the synthesis of hippuric acid. Salomon (8) isolated hippuric acid from the blood of nephrectomized rabbits. In 1911, Friedmann and Tachau (9) found hippuric acid after perfusing a rabbit liver with blood containing sodium benzoate. Significantly, the addition of glycine did not appear to increase the production of hippuric acid.

Snapper and Grünbaum (10) made the interesting observation that phenylpropionic acid and phenylvaleric acid when perfused through dog kidney yielded hippuric acid, while phenylacetic acid and phenylbutyric acid gave phenylaceturic acid.

While the bulk of the evidence is in favor of the hypothesis that the synthesis of hippuric acid in the dog occurs only in the kidney, it was felt that the problem is of such profound theoretical importance that further study seems justified.

## EXPERIMENTAL

*Analytical Methods*

*Free Benzoic Acid in Blood*—Defibrinated blood was treated by the original Folin-Wu method for the removal of blood proteins. The filtrate, after concentration by distillation under reduced pressure to approximately 40 cc., was made acid to Congo red and extracted with three 25 cc. portions of toluene. The combined fractions of toluene were washed with a slightly acidified saturated solution of sodium chloride, filtered through a dry filter, and titrated with 0.1 N sodium alcoholate, phenolphthalein being used as indicator.

*Hippuric Acid in Blood*—The acidified filtrate after extraction with toluene was divided into 10 or 15 cc. portions and extracted with ether in a continuous extractor. On boiling off the ether, hippuric acid when present could easily be detected by its characteristically high refractive and glistening needle crystals. The hydrolysis of hippuric acid was effected with 20 per cent hydrochloric acid. The determination of glycine by the formol titration was the same as in previous studies (11). The reliability of the method is attested by the fact that normal blood shows no hippuric acid and that on mixing 100 mg. of hippuric acid with 100 cc. of blood a recovery of over 80 per cent can be obtained.

*Hippuric Acid in Liver, Muscles, and Other Tissues*—The tissue immediately after removal was passed through a meat grinder and extracted with boiling water. The aqueous extract was concentrated by boiling and then treated with 3 volumes of alcohol. The resulting precipitate was removed by centrifuging, and the clear alcoholic solution evaporated on a water bath to a thick syrup which was dissolved in a small amount of hot water, and made acid to Congo red. The precipitate which formed was removed by centrifuging, and the clear supernatant solution extracted with ether in a continuous extractor. The determination of hippuric acid was the same as before. On mixing 100 mg. of hippuric acid with one-half of a ground liver, a recovery of about 50 per cent was obtained. In no experiment was either hippuric acid or phenylaceturic acid found; indicating that even if present the amount must be exceedingly small.

## DISCUSSION

No synthesis of hippuric acid could be demonstrated in a nephrectomized dog. This is in conformity with the observations of Bunge and Schmiedeberg and others, and furnishes additional evidence for the conclusion that in the dog the synthesis of hippuric acid occurs only in the kidney. The validity of this conclusion is especially well illustrated by a comparison of the results obtained on two dogs (Dogs 3 and 6) one with both kidneys removed, the other with ureters tied. After each had been given sodium benzoate and excess glycine intravenously, no hippuric acid could be found in the nephrectomized dog, but 81 mg. were found in the blood of the dog with the kidneys intact, but the ureters tied.

It will be noted that the concentration of hippuric acid in the blood is not only strikingly small, but also that it does not progressively increase even when the ureters are tied. This suggests that an equilibrium exists between the rate of synthesis of hippuric acid and its hydrolysis. It was previously shown that the conjugation of benzoic acid can best be explained by applying the law of mass action (12). The two basic equations are:

Benzoic acid + glycine  $\rightleftharpoons$  benzoylglycine (hippuric acid) + water

Benzoic acid + glycuronic acid  $\rightleftharpoons$  glycuronic acid monobenzoate + water

Since the ligation of the ureters stops the excretion of hippuric acid and glycuronic acid monobenzoate, these two products will tend to accumulate until the respective speeds of their hydrolysis will equal the speeds of synthesis when a condition of equilibrium will be established. Thus, it can readily be seen that hippuric acid cannot keep on accumulating in the blood but will reach a fixed maximum concentration which is necessarily small, since hippuric acid is present not only in the blood but is distributed through the other tissues of the body.

When the kidneys are removed, the mechanism for the synthesis of hippuric acid is destroyed, but the organism still possesses the power to hydrolyze hippuric acid, for apparently the hydrolytic enzyme is not localized in any one organ. Theoretically one can predict that on injecting hippuric acid, hydrolysis will begin immediately and go to completion since the mechanism for the reverse or synthetic process is absent. Ultimately only free benzoic acid and glycuronic acid monobenzoate should be found. This is

borne out by actual experiments. In one nephrectomized dog 2 gm. of hippuric acid given intravenously disappeared completely in 24 hours, and both free benzoic acid and glycuronic acid monobenzoate could be detected in the blood stream (Dog 7).

On feeding sodium benzoate to a nephrectomized rabbit, hippuric acid was readily isolated from the blood, liver, and muscles. This is in agreement with the observations of Salomon, and constitutes conclusive proof that the synthesis of hippuric acid in the rabbit is not, as in the dog, localized in the kidney. Friedmann and Tachau have demonstrated by means of perfusion experiments that the synthesis can take place in the liver, but it is not known whether other organs can also produce hippuric acid.

Phenylaceturic acid was definitely isolated from the blood of a nephrectomized dog following the injection of phenylacetic acid (Dog 9). This proves that the synthesis of phenylaceturic acid unlike that of hippuric acid does not take place solely in the kidney. It is even probable that the kidney is not even the main site of the synthesis in the normal animal, for on feeding phenylacetic acid and excess glycine, phenylaceturic acid can be found in the blood (Dog 10), indicating that the synthesis has been effected before the phenylacetic acid reaches the kidney. Hippuric acid, in agreement with the observations of Meissner and Shepard, is consistently absent from the blood (Dog 11) unless it is dammed back from the kidney, which occurs when the ureters are ligated.

Since a nephrectomized dog can produce phenylaceturic acid but not hippuric acid, it must be concluded that these two compounds require different enzymes for their synthesis. For the sake of convenience, the enzyme which is responsible for the conjugation of glycine with benzoic acid is named *hippuricase*, while the one which effects the union of glycine with phenylacetic acid is designated *phenaceturicase*. No logical explanation for their peculiar distribution can be given. In the dog *hippuricase* is present only in the kidney, while in the rabbit it is present in the liver and possibly in the kidney and other organs. *Phenaceturicase*, on the other hand, appears to be present both in the liver and kidney of the dog. In the human the problem is more complicated, for phenylacetic acid is conjugated with glutamine (13), while some of the substituted phenylacetic acids are combined with glycine (14).

The enzyme commonly called *histozyme*, which hydrolyzes hip-

puric acid, is found in various organs of the dog. It seems quite definite that this enzyme is entirely distinct from the synthetic enzyme, hippuricase. It is also doubtful whether true histozyne can hydrolyze phenylaceturic acid.

One cannot regard the presence of the conjugating enzymes in the kidney as merely accidental; nor can one set aside as incidental the fact that the higher phenyl-substituted aliphatic acids can undergo  $\beta$ -oxidation within this organ as was shown by Snapper and Grünbaum. These are probably important facts that must be properly evaluated in attempting to arrive at a comprehensive understanding of the function of the kidney.

#### SUMMARY

No hippuric acid was detected in the blood or liver of nephrectomized dogs that had been given sodium benzoate and glycine, but was found when the ureters were ligated.

On feeding sodium benzoate to nephrectomized rabbits, hippuric acid was found in the blood, liver, and muscles.

After injecting phenylacetic acid into a nephrectomized dog, phenylaceturic acid was isolated from the blood and definitely identified.

It was found that on injecting hippuric acid into nephrectomized dogs the compound gradually disappeared from the blood stream and free benzoic acid and glycuronic acid monobenzoate appeared. The data obtained on several dogs and a rabbit are given in the accompanying protocols.

#### CONCLUSIONS

The synthesis of hippuric acid is effected by an enzyme, hippuricase, which in the dog is apparently present only in the kidney, whereas the production of phenylaceturic acid is brought about by a different enzyme, designated phenaceturicase, which occurs not only in the kidney but also in some other organs. In the rabbit hippuricase is not localized in the kidney, since the nephrectomized animal can still synthesize hippuric acid.

The enzyme, histozyne, which hydrolyzes hippuric acid is distinct from hippuricase, since the nephrectomized dog can still hydrolyze but cannot synthesize hippuric acid.

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## PROTOCOLS

All operations were performed under ether anesthesia. Benzoic acid, phenylacetic acid, and hippuric acid were always given as the sodium salt.

*Fate of Benzoic Acid in Nephrectomized Dogs*

Dog 1, weight 22 kilos. June 3, 10.00 a.m., 15 gm. of gelatin given by stomach tube; 11.00 a.m., bilateral nephrectomy; 11.30 a.m., 3 gm. of benzoic acid given intravenously; 7.00 p.m., 1 gm. of benzoic acid and 1 gm. of glycine fed. June 4, 9.30 a.m., 0.5 gm. of benzoic acid and 3 gm. of gelatin fed; 11.00 a.m., animal exsanguinated. 1000 cc. of blood obtained. Benzoic acid combined as hippuric acid found in total blood, 0.015 gm.

All attempts to isolate hippuric acid failed. The recorded analysis although expressed as hippuric acid probably represents impurities such as phenylacetic acid and *p*-hydroxyphenylacetic acid.

Dog 2, weight 13 kilos. October 1, 11.00 a.m., bilateral nephrectomy. October 2, 10.00 a.m., 2 gm. of benzoic acid, 15 gm. of gelatin, and 150 cc. of milk fed; 12.00 m., 0.5 gm. of benzoic acid, 2.5 gm. of gelatin, and 75 cc. of milk fed; 2.00 p.m., animal exsanguinated. 550 cc. of blood obtained. Benzoic acid combined as hippuric acid found in total blood, 0.008 gm.

Dog 3, weight 8.5 kilos. October 8, 11.00 a.m., bilateral nephrectomy; 11.30 a.m., 2 gm. of benzoic acid and 5 gm. of glycine given intravenously; 2.30 p.m., animal exsanguinated. 250 cc. of blood obtained. Benzoic acid combined as hippuric acid found in total blood, 0.003 gm.

Dog 4, weight 12 kilos. October 19, 10.00 a.m., bilateral nephrectomy. October 20, 10.00 a.m., 2 gm. of benzoic acid, 15 gm. of gelatin, and 150 cc.



of milk fed; 12.30 p.m., 0.5 gm. of benzoic acid and 5 gm. of gelatin fed; 4.30 p.m., animal exsanguinated. 400 cc. of blood obtained. Benzoic acid combined as hippuric acid found in total blood, 0.008 gm.

In none of these four dogs was it possible to find or isolate crystals of hippuric acid by extracting the concentrated blood filtrate with ether.

#### *Fate of Benzoic Acid in Dogs with Ureters Ligated*

Dog 5, weight 8.5 kilos. June 22, 10.00 a.m., 15 gm. of gelatin given by stomach tube; 11.00 a.m., both ureters ligated; 11.30 a.m., 3 gm. of benzoic acid given intravenously; 7.30 p.m., 1 gm. of benzoic acid given orally. June 23, 10.00 a.m., animal exsanguinated. 425 cc. of blood obtained. Benzoic acid combined as hippuric acid found in total blood, 0.029 gm.

Dog 6, weight 9 kilos. October 5, 11.30 a.m., both ureters ligated; 12.30 p.m., 2 gm. of benzoic acid and 3 gm. of glycine given intravenously; 2.00 p.m., 70 cc. of blood withdrawn. Benzoic acid combined as hippuric acid found in total calculated volume of blood (470 cc.), 0.059 gm. October 6, 10.00 a.m., animal exsanguinated. 400 cc. of blood obtained. Benzoic acid combined as hippuric acid found in total blood, 0.034 gm.

Crystals of hippuric acid were found in the ether extract of the blood filtrate of both specimens.

#### *Fate of Injected Hippuric Acid in Nephrectomized Dogs*

Dog 7, weight 7 kilos. June 25, 11.00 a.m., bilateral nephrectomy; 11.30 a.m., 2 gm. of hippuric acid given intravenously. June 26, 11.00 a.m., animal exsanguinated. 175 cc. of blood obtained. Benzoic acid combined as hippuric acid found in total blood, 0.003 gm.

Free benzoic acid was present and the naphthoresorcinol test for glycuronic acid was strongly positive.

Dog 8, weight 12 kilos. October 13, 10.00 a.m., bilateral nephrectomy; 11.00 a.m., 3 gm. of hippuric acid given intravenously; 12.00 m., 25 cc. of blood withdrawn. Benzoic acid combined as hippuric acid found in total calculated volume of blood (325 cc.), 0.033 gm. Crystals of hippuric acid appeared in the ether extract. October 14, 10.30 a.m., animal exsanguinated. 300 cc. of blood obtained. Benzoic acid combined as hippuric acid found in total blood, 0.018 gm. No crystals of hippuric acid were found in the ether extract.

Free benzoic acid was found in the blood and liver.

#### *Fate of Phenylacetic Acid in a Nephrectomized Dog*

Dog 9, weight 10 kilos. June 26, 10.00 a.m., 15 gm. of gelatin given by stomach tube; 11.00 a.m., bilateral nephrectomy; 12.00 m., 3 gm. of phenylacetic acid given intravenously. June 27, 10.00 a.m., animal exsanguinated. 570 cc. of blood obtained. Phenylacetic acid combined as phenylaceturic acid found in total blood, 0.218 gm.

From the filtrate of 350 cc. of blood, 0.065 gm. of pure phenylaceturic acid was isolated. M.p. 141° uncorrected.

*Presence of Phenylaceturic Acid and Hippuric Acid in Blood*

Dog 10, weight 9 kilos. February 24, 2.30 p.m., 6 gm. of phenylacetic acid and 15 gm. of gelatin given by stomach tube; 6.30 p.m., animal exsanguinated. 450 cc. of blood obtained. Phenylacetic acid combined as phenylaceturic acid found in total blood, 0.062 gm.

Dog 11, weight 9 kilos. January 24, 10.00 a.m., 5 gm. of benzoic acid and 10 gm. of gelatin given by stomach tube; 2.00 p.m., animal exsanguinated. 400 cc. of blood obtained. Benzoic acid combined as hippuric acid found in total blood, 0.0 gm.

*Fate of Benzoic Acid in Nephrectomized Rabbit*

Rabbit 1, weight 2.25 kilos. January 16, 10.30 a.m., bilateral nephrectomy; 10.45 a.m., 0.25 gm. of benzoic acid and 0.5 gm. of glycine injected intravenously; 11.00 a.m., 1.5 gm. of benzoic acid and 15 gm. of gelatin given by stomach tube; 8.00 p.m., animal exsanguinated. Benzoic acid combined as hippuric acid found in 50 cc. of blood was 0.057 gm.; in 45 gm. of liver, 0.016 gm.; and in 500 gm. of muscle, 0.063 gm.

The characteristic crystals of hippuric acid appeared in the ether extract of each of the reported analyses.



# THE RELATIONSHIP BETWEEN CHEMICAL STRUCTURE AND PHYSIOLOGICAL RESPONSE

## I. THE CONJUGATION OF SUBSTITUTED BENZOIC ACIDS\*

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A quantitative study of the conjugation of various substituted benzoic acids was undertaken with the object of determining the influence of different chemical groups when occupying various positions in the molecule. It was hoped that such a study might yield new information concerning the relationship between chemical structure and physiological response.

The literature on the conjugation of aromatic acids is unsatisfactory. Nearly all workers have either underestimated or entirely ignored the possibility that aromatic acids may be combined with glycuronic acid. Even as late as 1929 two investigators (1) concluded that *p*-chlorobenzoic acid caused glycosuria because, after feeding this compound to dogs, the urine reduced Fehling's solution. The seriousness of overlooking the conjugation with glycuronic acid is obvious, for in many instances it is quantitatively far more important than the combination with glycine. This oversight, however, has led to a second error which is equally grave. The glycuronic acid union with an aromatic acid is comparatively labile and unless special precautions are taken, it is readily hydrolyzed. Since most investigators were not cognizant of the presence of an unstable conjugated product, and since almost every one concentrated the urine by heating before attempting the isolation of the conjugated products, they

\* This work was aided by a gift of Mrs. John L. Given in support of surgical research.

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TABLE I  
Glycine Conjugation of Substituted Benzoic Acids

Compound studied	Recorded m p of hippuric acid	Remarks	Author
	°C.		
<i>p</i> -Toluic acid	161-162	In man	Kraut (2); Gleditsch and Moeller (3)
<i>m</i> -Toluic "	162-165	" " Also found after feeding <i>m</i> -xylene to dogs	Gleditsch and Moeller (3)
<i>o</i> -Toluic "	139	In man. Excretion of much uncombined acid noted	" "
<i>p</i> -Fluorobenzoic acid	161-161.5	In dogs	Coppola (4)
<i>m</i> -Fluorobenzoic "	152-153	" "	"
<i>o</i> -Fluorobenzoic "	121	" "	"
<i>p</i> -Chlorobenzoic "	143	" "	Hildebrandt (5)
	143*	" " not in rabbits	Novello, Miriam, and Sherwin (6); Schübel and Manger (1)
		In dogs	
<i>m</i> -Chlorobenzoic acid		" man, only calcium salt obtained	Graebe and Schultzen (7)
		After feeding <i>m</i> -toluene to rabbits and dogs; not obtained pure	Hildebrandt
	143-144*	No conjugation observed in dogs and rabbits	Novello <i>et al.</i>
<i>o</i> -Chlorobenzoic acid		As potassium salt after feeding <i>o</i> -chlorotoluene to dogs	Hildebrandt
	176*	No conjugation observed in dogs and rabbits	Novello <i>et al.</i>
<i>p</i> -Bromobenzoic acid		After feeding <i>p</i> -bromotoluene to dogs; not obtained pure	Preusse (8)

TABLE I—*Continued*

Compound studied	Recorded m. p. of hippuric acid	Remarks	Author
	°C.		
<i>p</i> -Bromobenzoic acid		After feeding <i>p</i> -bromotoluene to rabbits	Hildebrandt
<i>m</i> -Bromobenzoic acid	159-162	In dogs and rabbits	Novello <i>et al.</i>
	183	" "	Hildebrandt
	146-147*	" " not in rabbits	Novello <i>et al.</i>
<i>o</i> -Bromobenzoic "	153	<i>o</i> -Bromotoluene completely converted to hippuric acid in rabbit	Hildebrandt
	192-193*	On feeding 6 gm. of acid, 5 gm. of <i>o</i> -bromohippuric acid obtained in dog; also in rabbit	Novello <i>et al.</i>
<i>p</i> -Iodobenzoic acid	188-190*	In dogs and rabbits	" " "
<i>m</i> -Iodobenzoic "	167-169*	Only impure product obtained	" " "
<i>o</i> -Iodobenzoic "	170*	After feeding 6 gm. of acid to dog, 2.5 gm. of <i>o</i> -iodohippuric acid obtained	" " "
<i>p</i> -Hydroxybenzoic acid		In dogs	Baumann and Herter (9)
		" man, at least partially	Schotten (10)
		No conjugation observed in man	Power and Sherwin (11)
		No conjugation observed in monkey	Sherwin (12)
<i>m</i> -Hydroxybenzoic acid		In dogs	Baumann and Herter
<i>o</i> -Hydroxybenzoic acid	160	" man	Bertagnini (13); extensive literature

TABLE I—*Continued*

Compound studied	Recorded m.p. of hippuric acid	Remarks	Author
	°C.		
<i>p</i> -Aminobenzoic acid		No conjugation observed in dogs	Hildebrandt
	199*	No conjugation observed in dogs or man	Muenzen, Cerecedo, and Sherwin (14)
<i>m</i> -Aminobenzoic acid	192	In rabbits and dogs	Salkowski (15)
		No conjugation observed in dogs	Hildebrandt
		No conjugation observed in dogs, rabbits, or man	Muenzen <i>et al.</i>
<i>o</i> -Aminobenzoic acid		No conjugation observed in dogs	Hildebrandt
		No conjugation observed in dogs, rabbits, or man	Muenzen <i>et al.</i>
<i>p</i> -Nitrobenzoic acid	129	In dogs	Jaffé (16)
		" dog after feeding <i>p</i> -nitrobenzaldehyde	Sieber and Smirnow (17)
		In man after eating <i>p</i> -nitrobenzaldehyde	Sherwin and Hynes (18)
<i>m</i> -Nitrobenzoic acid		In dogs after feeding <i>m</i> -nitrobenzaldehyde	Sieber and Smirnow
	165	In rabbits and dogs after feeding <i>m</i> -nitrobenzaldehyde	Cohn (19)
	160–162	In man after eating <i>m</i> -nitrobenzaldehyde	Sherwin and Hynes
<i>o</i> -Nitrobenzoic acid		No conjugation after feeding dogs <i>o</i> -nitrotoluene	Jaffé (20)
		Same as above after feeding <i>o</i> -nitrobenzaldehyde	Sieber and Smirnow
		Same as above	Cohn (19)
		" " "	Sherwin and Hynes

TABLE I—*Concluded*

Compound studied	Recorded m.p. of hippuric acid	Remarks	Author
	°C.		
$\alpha$ -Naphthoic acid		In dogs, not in rabbits	Cohn (21)
		Not in man	Nencki (22)
$\beta$ -Naphthoic “		In rabbits, not in dogs	Cohn (21)
<i>p</i> -Chlorophenylacetic acid	135	In man, dogs, and rabbits	Cerecedo and Sherwin (23)
Mandelic acid		In man	Graebe and Schultzen
		Not in dogs	Schotten (24)
		“ “ “	Knoop (25)

\* Melting point of synthetically prepared compound.

inadvertently hydrolyzed at least a portion of the glycuronic acid complex and thus obtained the free acid. The reported findings of uncombined acids are, therefore, almost meaningless, since it is difficult, in fact impossible, to know whether the particular acid studied was actually excreted uncombined or was hydrolyzed in the isolation procedure.

Nearly all of the work on the substituted aromatic acids is limited to their conjugation with glycine. Unfortunately, the lack of satisfactory analytical methods and the employment at times of haphazard isolation procedures have given rise to serious inaccuracies. For the sake of compactness, references to the glycine conjugation of substituted benzoic acids are given in Table I.

Jaffé, whose work stands out even among that of his illustrious contemporaries for its accuracy and keenness, commented on the fact that no *o*-nitrohippuric acid could be found in the urine after feeding *o*-nitrotoluene to dogs, whereas if the meta and para compounds were fed, the corresponding hippuric acids could easily be isolated (20). Later workers (17–19) have corroborated his findings, and Sherwin and Hynes observed further that *o*-nitrobenzaldehyde was much more toxic than the para and meta isomers. Jaffé obtained a compound in crystalline form after



TABLE II  
Conjugation of Substituted Benzoic Acids

Compound	Ionization constant 25°	Excretion of compound (expressed in terms of benzoic acid)			Excess glycine supplied* Excretion of compound (in terms of benzoic acid)																																																																																																																																																																																																																																																																																																																																																																																																																																																							
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
















	<i>m</i> -Chlorobenzoic acid	$1.6 \times 10^{-4}$	0.31	0.34	0.61	0.48	0.69	0.68	0.63	0.44	0.53	0.44	0.22			
	<i>o</i> -Chlorobenzoic acid	$1.3 \times 10^{-3}$	0.38	0.27	0.12	0.11	0.27	0.54	0.50	0.64	0.19	0.15	0.31	0.52	0.40	0.36
	<i>p</i> -Bromobenzoic acid		0.55	0.42	0.42	0.24	0.35	0.50	0.90	0.55	0.43	0.40	0.19	0.41		
	<i>m</i> -Bromobenzoic acid	$1.4 \times 10^{-4}$	0.35	0.36	0.96	0.38	0.82	0.76	0.70	0.42	0.93	0.33				
	<i>o</i> -Bromobenzoic acid	$1.5 \times 10^{-3}$	0.36	0.05	0.09	0.09	0.20	0.58	0.50	0.57	0.09	0.06	0.11	0.58	0.42	0.57
	<i>p</i> -Iodobenzoic acid†		0.25	0.21	0.54	N.R.†	N.R.	0.46	0.32	0.67	N.R.					

TABLE II—Continued

Compound	Ionisation constant 25°	Excretion of compound (expressed in terms of benzoic acid)												Excess glycine supplied* Excretion of compound (in terms of benzoic acid)			
		Free			Glycine			Glycuronic acid			Glycine			Glycine			Glycuronic acid
		6 hrs.	12 hrs.	24 hrs.	6 hrs.	12 hrs.	24 hrs.	6 hrs.	12 hrs.	24 hrs.	6 hrs.	12 hrs.	24 hrs.	6 hrs.	12 hrs.	24 hrs.	
 <i>o</i> -Iodobenzoic acid† $1.4 \times 10^{-3}$			0.12	0.02	0.09	0.47	0.41	0.40	0.08	0.03				0.61	0.41	0.43	
 <i>p</i> -Hydroxybenzoic acid $2.8 \times 10^{-5}$			0.29	0.18	0.67	0.26			0.31	0.13				0.71	0.18		
 <i>m</i> -Hydroxybenzoic acid $8.7 \times 10^{-5}$			0.33	0.15	0.82	0.35			0.50					0.89	0.25		
 <i>o</i> -Hydroxybenzoic acid $1.0 \times 10^{-3}$		0.09	0.07	0.08	0.07	0.17	0.48	0.50	0.80								
 <i>p</i> -Aminobenzoic acid					1.15	0.65	0.35							1.22	0.38		

	<i>m</i> -Aminobenzoic acid									0.87	0.62						0.80	0.42	
	<i>o</i> -Aminobenzoic acid									1.67	0.50						1.60	0.77	
	<i>p</i> -Nitrobenzoic acid	$4.0 \times 10^{-4}$				0.37	0.36			0.34	0.37			0.44	0.23	0.74	0.42	0.33	0.50
	<i>m</i> -Nitrobenzoic acid	$3.4 \times 10^{-4}$				0.01	0.06	0.31	0.25	0.70	0.36	0.36	0.55	0.38	0.43	0.77	0.50	0.52	0.48
	<i>o</i> -Nitrobenzoic acid	$6.2 \times 10^{-3}$				2.28	0.33	0.11	0.05	0.24	0.26	0.26	0.31	0.00	0.10		0.24	0.18	0.21
	$\alpha$ -Naphthoic acid	$2.0 \times 10^{-4}$				0.02	0.01	0.15	0.13	0.17	0.55	0.64	0.45						



feeding *o*-nitrotoluene, which from his description appears to have been a glycuronic acid conjugation product, but since glycuronic acid had not been definitely recognized at that time, he failed to identify the compound. Later Jaffé (26) isolated the glycuronic acid compound of *p*-dimethylaminobenzoic acid. There are a few other scattered reports of glycuronic acid compounds of substituted aromatic acids. Baldoni (27) reported isolating a glycuronic acid complex of salicylic acid. Mitsuba and Ichihara (28) found a large excretion of combined glycuronic acid when *o*-aminobenzoic acid was fed to dogs and rabbits. Muenzen, Cerecedo, and Sherwin (14), on the other hand, reported that no reducing action of the urine was found after feeding this drug to dogs. Miriam, Wolf, and Sherwin (29) found that diphenylacetic acid was conjugated with glycuronic acid in man, in the dog, and in the rabbit.

#### EXPERIMENTAL

All of the compounds studied were obtained from the Eastman Kodak Company, except *o*-chlorophenylacetic acid, which was prepared from *o*-chlorobenzyl chloride. All experiments, with the few exceptions noted in Table II, were carried out on two dogs which were of approximately equal size and weight. It was surprising how closely the results of any particular experiment on one dog could be duplicated on the second dog.

The compounds were neutralized and incorporated with a diet consisting of casein, sucrose, lard, and bone ash. Feeding experiments were carried out only every other day and frequently longer rest periods were allowed. Ordinary table scraps were fed on the days when no drug was given. In an attempt to get comparable results, the equivalent of 3 gm. of benzoic acid of the particular compound studied was fed. All the substances were well tolerated, the dogs maintained their appetites, lost no weight, and at the end of the experiment had no demonstrable kidney or liver damage.

#### *Analytical Methods*

The methods previously developed for the determination of hippuric acid and glycuronic acid monobenzoate are applicable, with minor modifications, for the determination of the glycine

and glycuronic acid compounds of most of the substituted benzoic acids. No satisfactory procedure for isolating or determining aminohippuric acids was found. The hydrolysis of hippuric acid is brought about by refluxing with 20 per cent hydrochloric acid instead of the concentrated acid. Since some of the substituted hippuric acids are fairly resistant to hydrolysis, it is best to reflux for at least 2 hours.

*Free or Uncombined Substituted Benzoic Acid*—A new method for determining free substituted benzoic acids had to be developed since many of the acids cannot be extracted directly from the urine with toluene or chloroform. Satisfactory results were obtained by the following procedure: 20 cc. of urine were made acid to Congo red, a few drops of 10 per cent sodium tungstate added, and extracted with ether for 90 minutes in a continuous extractor. The ether was boiled off and the flask left on the water bath until the residue was dry. It was then extracted with two 25 cc. portions of boiling toluene which were passed through a dry filter and titrated with 0.1 N sodium alcoholate solution, phenolphthalein being used as indicator. The residue remaining after the extraction with toluene can be used for the determination or isolation of the hippuric acid present. Most of the meta- and para-substituted hippuric acids as well as o-chlorohippuric acid were isolated in this manner.

#### DISCUSSION

It is generally accepted that the various conjugation processes of the body are due to enzyme action. In the preceding paper it was shown that hippuric acid and phenylaceturic acid require different enzymes for their synthesis. Apparently benzoic acid and phenylacetic acid present two different stereochemical configurations or patterns for enzyme action. From their formulæ,  $\text{PhCOOH}$  and  $\text{PhCH}_2\text{COOH}$ , it is to be concluded that it must be the configuration of the side chain upon which the action of the enzyme is apparently chiefly dependent; and it seems, therefore, reasonable to conjecture that substitution in the aromatic nucleus will not basically alter the essential pattern or configuration, but that such changes in the molecule might nevertheless definitely influence, especially from the quantitative point of view,

the activity of the enzyme. On comparing the conjugation of benzoic acid and *p*-chlorobenzoic acid as to rate of excretion and the effect of feeding excess glycine, one finds a fairly close agreement, and it can be concluded that both conjugations are brought about by the same enzyme. A similar correspondence is found for phenylacetic acid and *o*-chlorophenylacetic acid. In the latter pair, the marked response to exogenous glycine is especially outstanding.

On inspecting the quantitative data on the conjugation of substituted benzoic acids, one outstanding fact immediately becomes evident. There is little or no conjugation of glycine with ortho-substituted benzoic acids. If the ortho position is occupied, the conjugation with glycine is so completely inhibited that it is difficult to find any hippuric acid. Only in the case of *o*-chlorobenzoic acid was it possible to isolate and identify the expected hippuric acid, although the formation of other ortho-substituted hippuric acids in minute amounts was indicated in several other instances. The effect appears to be independent of the type of group, for the halogens, the nitro, the hydroxy, and the methyl groups produce the same impeding influence. Because of the lack of a satisfactory method for determining aminohippuric acids, it cannot be stated with certainty whether the amino group also hinders the conjugation with glycine. There is, however, suggestive evidence that it does. It will be observed that feeding an excess of glycine produces a decrease in the excretion of glycuronic acid only if the particular acid is conjugated with glycine. Such a decrease occurs with *m*- and *p*-aminobenzoic acid but not with the ortho acid.

Of particular interest and perhaps of practical importance is the fact that  $\alpha$ -naphthoic acid behaves like an ortho-substituted benzoic acid in that it undergoes little if any conjugation with glycine. Apparently, the second benzene ring has the same effect as substitution in the ortho position, and it should be noted that the carbon atom that is ortho to the carboxyl group and common to both benzene rings contains no replaceable hydrogen. The impeding influence of ortho substitution occurs only if the carboxyl group is attached directly to the ring, for *o*-chlorophenylacetic acid is readily conjugated with glycine. The fact that mandelic acid is not conjugated with glycine suggests that substitution



on the  $\alpha$ -carbon atom of the aliphatic chain may perhaps also produce steric hindrance.

The results of Hildebrandt (5) and of Novello, Miriam, and Sherwin (6), who found a large excretion of *o*-bromohippuric acid, are difficult to explain. Even after feeding excess of glycine with *o*-bromobenzoic acid, no *o*-bromohippuric acid could be isolated, nor did the analytical results show more than a trace. On the other hand, *o*-chlorohippuric acid, which Novello, Miriam, and Sherwin failed to find, was easily isolated and identified. In fact its perfectly shaped diamond crystals make its recognition extremely easy.

That substitution in the ortho position offers peculiar difficulties for enzyme action is further demonstrated by the work of Balls (30) who found that whereas the glycyl *m*- and *p*-aminobenzoic acids are readily hydrolyzed, the ortho compound completely resists enzyme action.

The conjugation of benzoic acid with glycuronic acid is greatly influenced by substitution in the ortho position, but in contrast to the conjugation with glycine, the nature of the substituting group is of utmost importance. Taking the conjugation of benzoic acid as standard, it was found that a neutral group such as the methyl exerted no definite effect; a relatively acidic group such as the hydroxy or the halogens caused a 50 per cent decrease in the glycuronic acid excretion; a strongly acidic group such as the nitro depressed the output of glycuronic acid even more; while a basic group such as the amino produced in marked contrast an increase of more than 50 per cent.

Substitution in the meta position also influences the glycuronic acid excretion but to a less extent, and the effect tends to be the opposite to that of ortho substitution. It will be noted that the hydroxy, chlorine, and bromine, all distinctly acid, when in the meta position will induce a larger output of glycuronic acid than when the same radical is in the ortho or para position. On the other hand, the basic amino group when in the meta position produces a decrease in the excretion of glycuronic acid as compared with its ortho and para isomers. It thus appears that an acid group in the meta position is somewhat equivalent to a positive group in the ortho position and *vice versa*. It is interesting that *o*-chlorophenylacetic acid behaves like a meta-substituted benzoic

acid, for it produces a larger output of conjugated glycuronic acid than phenylacetic acid.

It seems quite definite that the strength of the acid constitutes one of the principal factors governing its conjugation with glycuronic acid. There is a fairly constant, though not absolute, correlation between the pK of an acid and the rate with which it is excreted combined with glycuronic acid.

In this discussion it is necessary to speak of the output or excretion rather than of the production of glycuronic acid, for it is not known whether the decreased excretion of glycuronic acid found, for example, in the case of *o*-chlorobenzoic acid is due to a delay in the synthesis or to a rapid oxidation of the glycuronic acid after the conjugation has taken place. There is a gradual accumulation of evidence showing that conjugated glycuronic acids can be burned in the body. It will be recalled that the author (31) recently found that menthol in the dog is presumably conjugated with glycuronic acid as indicated by the fact that when menthol is fed with sodium benzoate it will decrease the output of glycuronic acid monobenzoate. Nevertheless, only a trace of menthol glycuronic acid appears in the urine, and even when menthol glycuronic acid is fed only a very small fraction is excreted. This indicates that menthol is first combined with glycuronic acid and then the conjugated product is completely burned. It seems improbable that glycuronic acid, a compound having a high caloric value, is intended primarily as an excretory product. It seems more likely that its conjugation may perhaps represent the preliminary step in the oxidation of the particular substance with which it is united, irrespective whether the linkage is of the glycosidic or the ester type. If the substance with which it is conjugated resists catabolism, only the glycuronic acid may suffer destruction, with the result that the original substance is again set free. This appears to occur with benzoic acid, for if glycuronic acid monobenzoate is injected intravenously, a small fraction of the benzoic acid will be excreted as hippuric acid.

The fact that the potential supply of glycuronic acid is unusually large and that the amount appearing in the urine may be no measure whatsoever of the quantity produced for conjugation, leads one to wonder whether the importance of glycuronic acid may not be underestimated. The possibility that the fatty acids

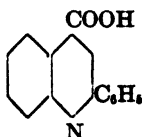
may at a stage in their metabolism unite with glycuronic acid offers a theory to explain how fats are burned in the fire of carbohydrates. The relationship between the  $pK$  of an acid and its conjugation with glycuronic acid, furthermore, suggests the possibility of a new type of mechanism by which the body may influence metabolism. It is interesting to note that the ionization constant of butyric acid is  $1.48 \times 10^{-5}$ , which comes within the range of the ionization constants of those substituted benzoic acids which are largely excreted combined with glycuronic acid; and, furthermore, that by the introduction of a negative group such as takes place in the conversion of butyric acid to acetoacetic acid a much stronger acid is produced. Whether these facts are of any moment or are merely incidental can only be decided by further study.

The excretion of free or uncombined acid was observed only with those benzoic acids which had an acid group in the ortho position. It was most marked in the case of *o*-nitrobenzoic acid. Whether this free acid represents a fraction which traverses the body without undergoing conjugation or which combines with glycuronic acid and later is again liberated by the oxidation of the glycuronic acid cannot be decided until the problem has been more intensively studied.

No attempt was made to make a complete study of the conjugation of the hydroxy- and the aminobenzoic acids. In the case of the hydroxybenzoic acids at least four different conjugated products are possible, for the hydroxy group can combine with sulfuric acid and with glycuronic acid while the carboxyl group can unite with either glycine or glycuronic acid. Both the *m*- and the *p*-hydroxybenzoic acids are conjugated with glycuronic acid through the carboxyl group and probably also through the hydroxy radical; and both acids are rapidly eliminated. The excretion of *o*-hydroxybenzoic acid, salicylic acid, in marked contrast is slow and prolonged. The finding that ortho substitution inhibits glycine conjugation and also decreases the glycuronic acid output, if the group is acid, has particular significance for arriving at a better understanding concerning the fate of salicylic acid in the body. It seems probable that salicylic acid passes through repeated cycles of metabolism. It is conceivable that glycuronic acid is combined with the phenolic group, then due

to the difficulty that the body has excreting ortho-substituted acids, there is enough delay to allow the glucuronic acid to be oxidized again with a reliberation of the phenolic group. Of all the substituted benzoic acids studied, salicylic acid appeared to be the most toxic. It readily induced vomiting, and in one dog caused a persistent polyurea which lasted over 1 week. A more comprehensive study of the conjugation of the hydroxybenzoic acids will be reported later.

The physiological importance of ortho substitution is suggested by two other drugs of current interest, namely cincophen and tri-*o*-cresylphosphate. The structure of cincophen



shows a carboxy group in essentially the same position as in  $\alpha$ -naphthoic acid, which as has been shown behaves like an ortho-substituted benzoic acid. Hitherto no explanation has been offered for the similarity in the pharmacological action between cincophen and salicylic acid, but from the observations made in the present study it can be seen that both compounds contain a carboxyl group which cannot freely be conjugated with glycine. A more complete study of this problem will be presented in a later paper.

The toxic action of tri-*o*-cresylphosphate, the chemical responsible for the so called ginger paralysis, is of particular interest as the *m*- and *p*-cresylphosphates do not produce the characteristic type of motor paralysis (32). Since *p*-cresol according to Preusse (8) is readily oxidized in the body to *p*-hydroxybenzoic acid, one can assume that a similar oxidation occurs in the case of tri-*p*-cresylphosphate and that the resulting phosphoric esters of *p*-hydroxybenzoic acid are readily detoxicated and eliminated by the organism. In the case of tri-*o*-cresylphosphate, however, even though oxidation of the methyl groups should occur, the body would still be confronted with the problem of handling an acid in which the carboxyl is ortho to an acidic group, a chemical combination which causes the organism much difficulty. Being unable to

excrete the compound promptly, the body is subject to a prolonged exposure to its toxic action.

#### SUMMARY

Substitution of benzoic acid in the ortho position greatly inhibits its conjugation with glycine, and this effect appears to be independent of the nature of the group.

The conjugation of benzoic acid with glycuronic acid is definitely influenced by substitution in the ortho position. An acidic group diminishes the output of the glycuronic acid compound; a basic group greatly increases the excretion; while a neutral group has little effect. Substitution in the meta position has a less pronounced effect and tends to be the opposite to that of ortho substitution, *i.e.* an acid group in the meta position will induce a larger excretion of conjugated glycuronic acid than when the same group is in the ortho or para position. There is a close relationship between the ionization constant of the substituted benzoic acid and the rate with which it is excreted combined with glycuronic acid.

The significance of the effect of ortho substitution is discussed and the importance of the present findings in developing a better understanding of the toxic and pharmacological action of such compounds as salicylic acid, cincophen, and tri-*o*-cresylphosphate is emphasized.

A new method for determining uncombined or free substituted benzoic acids in urine is described.

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## THE METABOLISM OF THE PHOSPHOLIPIDS

### V. THE RELATIONSHIP BETWEEN THE AMOUNT OF FAT INGESTED AND THE DEGREE OF UNSATURATION OF THE PHOSPHOLIPIDS AND NEUTRAL FAT IN THE TISSUES OF THE RAT

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#### INTRODUCTION

In a recent paper dealing with the comparative influence of various fats on the degree of unsaturation of the phospholipids in the tissues of the rat (Sinclair, 1931), it was shown that if rats are raised on a synthetic diet which is relatively poor in fat, the phospholipid fatty acids have a uniformly low iodine number. If to this fat-poor diet fat is added, the iodine number of the phospholipid fatty acids is always increased, the exact level being characteristic of the particular fat which is fed. In this present paper are presented the results of a study of the relationship between the *amount* of fat fed and the iodine number of the phospholipid fatty acids in the entire animal, the carcass, and the liver. In addition data have also been obtained on the comparative effect of various amounts of food fat on the degree of unsaturation of the neutral fat.

#### EXPERIMENTAL

##### *Procedure*

Young healthy rats of both sexes, weighing between 30 and 40 gm., were placed individually in cages of  $\frac{1}{2}$  inch wire mesh set on 2 inch legs. Tap water was provided *ad libitum*. The diets, of the so called synthetic type, consisted of casein, cane-sugar, salt



mixture,<sup>1</sup> dried yeast, and oscodal,<sup>2</sup> supplemented with various amounts of fat. The fat was either fed mixed with the remainder of the ration, or given separately in small dishes with the dried yeast, or administered *per os* by means of a dropping pipette. Since the diets differed from one experiment to another, the details will be given with the description of each experiment. When body weights of from 75 gm. to above 200 gm. had been reached, individual rats were removed and analyzed for the amount and iodine number of the tissue lipids. The experimental procedure employed has already been described in an earlier paper (Sinclair, 1930,a).

### Results

*A. Influence of Various Amounts of Cod Liver Oil on Degree of Unsaturation of Lipids in the Entire Body of the Rat*—In this first experiment dealing with the influence of small amounts of fat on the composition of the phospholipids, the rats were fed *ad libitum* on a "fat-free" basic diet consisting of 24 per cent of alcohol-extracted casein, 72.1 per cent of cane-sugar, and 3.9 per cent of salt mixture.<sup>3</sup> In addition each rat received daily 0.65 gm. of ether-extracted dried yeast, from 1 to 5 mg. of oscodal, and from 0 to 280 mg. of cod liver oil, all given together in a small dish. The cod liver oil was measured out in drops from a calibrated pipette. In Fig. 1 these daily doses of oil have been translated into mg. by taking the average of eight weighings of the 3, 6, 9, 12, and 15 drops.

In Fig. 1 the data obtained for the iodine numbers of the fatty acids of the phospholipids and neutral fat in the entire bodies are plotted against the average daily intake of cod liver oil. The heavy points on the graph are the averages of the values for the three or four rats which received the same amount of oil each day. The weights of the individual rats in each group at death are indicated by symbols in the legend. The curve showing the relationship between the iodine number of the phospholipid

<sup>1</sup> McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, **33**, 63 (1918). All diets contained 1 gm. of salt mixture per 100 calories.

<sup>2</sup> A concentrate containing vitamins A and D, kindly supplied by the H. A. Metz Laboratories, Inc., through the courtesy of Dr. H. E. Dubin.

<sup>3</sup> This diet was copied after Diet 550 of Burr and Burr (1929).

fatty acids and the amount of cod liver oil fed per day was plotted from the equation

$$\text{Iodine No.} = 13.63 \log_{10} \text{amount of cod liver oil} + 102.2$$

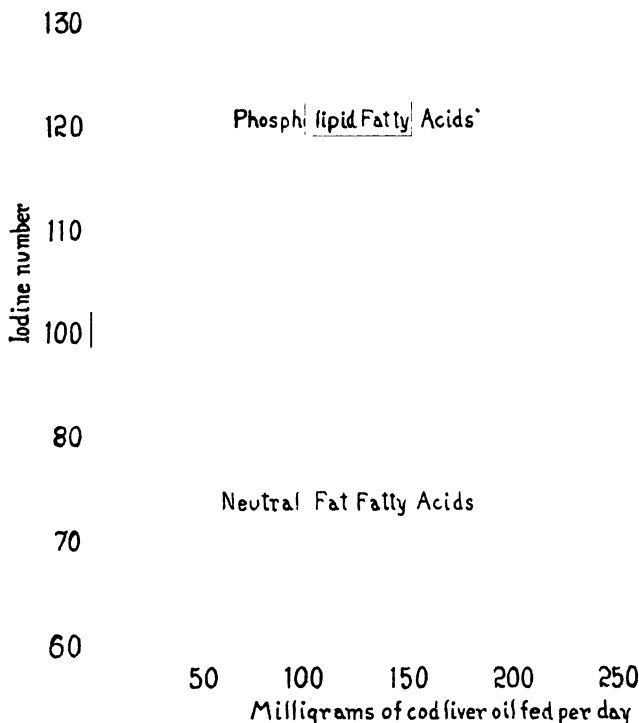


FIG. 1. Showing the relationship between the iodine numbers of the phospholipid and neutral fat fatty acids in the entire bodies of rats and the amount of cod liver oil ingested per day. The weights of the rats in each group when killed were as follows: +, 72 to 74 gm.; ○, 99 to 101 gm.; △, 150 to 156 gm.; ■, 202 gm.

which was derived from the experimental data by the method of least squares.<sup>4</sup> It is scarcely necessary to point out that this

<sup>4</sup> The author is indebted to Professor C. W. Watkeys, of the Mathematics Department of The University of Rochester, for advice as to the mathematical treatment of these data.

linear relationship between the iodine number of the phospholipid fatty acids and the logarithm of the amount of cod liver oil fed each day does not hold when no oil is fed. In such a case the iodine number of the phospholipid fatty acids (101.2 being the average of thirteen rats raised on our "fat-free" Diet 20<sup>5</sup>) is a function of the capacity of the rat to synthesize and to desaturate fatty acids.

The following highly significant facts are brought out by this experiment: (1) The presence in the diet of small amounts of cod liver oil results in the synthesis of tissue phospholipids which are much more highly unsaturated than those of rats raised on the fat-free diet alone. For example, the daily ingestion of about 100 mg. of cod liver oil by a growing rat will cause a 30 per cent increase in the iodine number of the phospholipid fatty acids over the level characteristic of the basic fat-free ration. (2) These small amounts of cod liver oil which have such a marked effect on the degree of unsaturation of the phospholipids have no apparent effect on that of the neutral fat. (3) If the amount of cod liver oil ingested daily by a rat remains constant, the degree of unsaturation of the tissue phospholipids also remains relatively constant despite the increase in body weight. This fact is adequately brought out by noting the range in the weights of the various animals in each group receiving the same amount of fat each day. (4) There is a very definite quantitative relationship between the iodine number of the phospholipid fatty acids in the tissues and the amount of cod liver oil in the diet.

*B. Relationship between Percentage of Fat in Diet and Degree of Unsaturation of Tissue Lipids*—In the experiment just described the rats were fed a certain constant amount of cod liver oil each day throughout the experimental period, and in most instances this daily dose was all consumed within a short period of time. It seemed reasonable that, as a result, the blood and tissue fluids probably contained cod liver oil fatty acids for but a few hours after the ingestion of the oil and that for the greater part of the day the circulating lipids were about the same as those of rats which were fed exclusively on the fat-free diet. Consequently it was thought that a relationship somewhat different from that

<sup>5</sup> The numbers refer to diets listed in Table I of a preceding paper (Sinclair, 1931).

shown in Fig. 1 might be exhibited if the cod liver oil was fed in such a way that it would be in the blood stream more or less continually throughout the day. To this end the cod liver oil was mixed with the main ration, the idea being that in this way the oil would enter the blood stream just as frequently as the rat ate.

Rats were raised on diets which were so made up that the casein (alcohol-extracted) provided 25 per cent of the calories, cod liver oil from 2.5 per cent to 40 per cent, and cane-sugar the remainder of the calories. Dried yeast was mixed with the ration on the basis of 2.5 gm. per 100 calories. The mixed rations were made up fresh each day. In other respects this second experiment was similar to the first, the three or four rats on the same diet being killed when the weights were approximately 75, 125, and 160 gm. Data were obtained on the iodine number of the fatty acids present in both the phospholipids and the neutral fat in the entire bodies. The results are set forth in Fig. 2, the iodine number being plotted against the percentage of the calories derived from cod liver oil. It is at once evident that the results conform in general with those given in Fig. 1. A very definite relationship exists between the iodine number of the phospholipid fatty acids and the percentage of fat calories in the diet. In this experiment no attempt has been made to derive the mathematical expression of this relationship, the curve in Fig. 2 having been placed purely by inspection.

Of the greatest significance is the further demonstration that small amounts of food fat exert a much more pronounced effect on the degree of unsaturation of the tissue phospholipids than on that of the depot fat. It may be seen that a further increase in the cod liver oil intake above 5 per cent of the total calories has a comparatively slight effect on the composition of the phospholipids, whereas the degree of unsaturation of the neutral fat is very markedly increased. This second experiment also offers further evidence that, provided the diet remains constant, the iodine numbers of both the phospholipid and neutral fat fatty acids also remain constant during the actively growing period of the rat's lifetime. In general there is a somewhat greater spread of values among the rats in the same group in the second experiment than in the first. This may have been due to either or both of two factors: the greater caloric and therefore fat intake of the

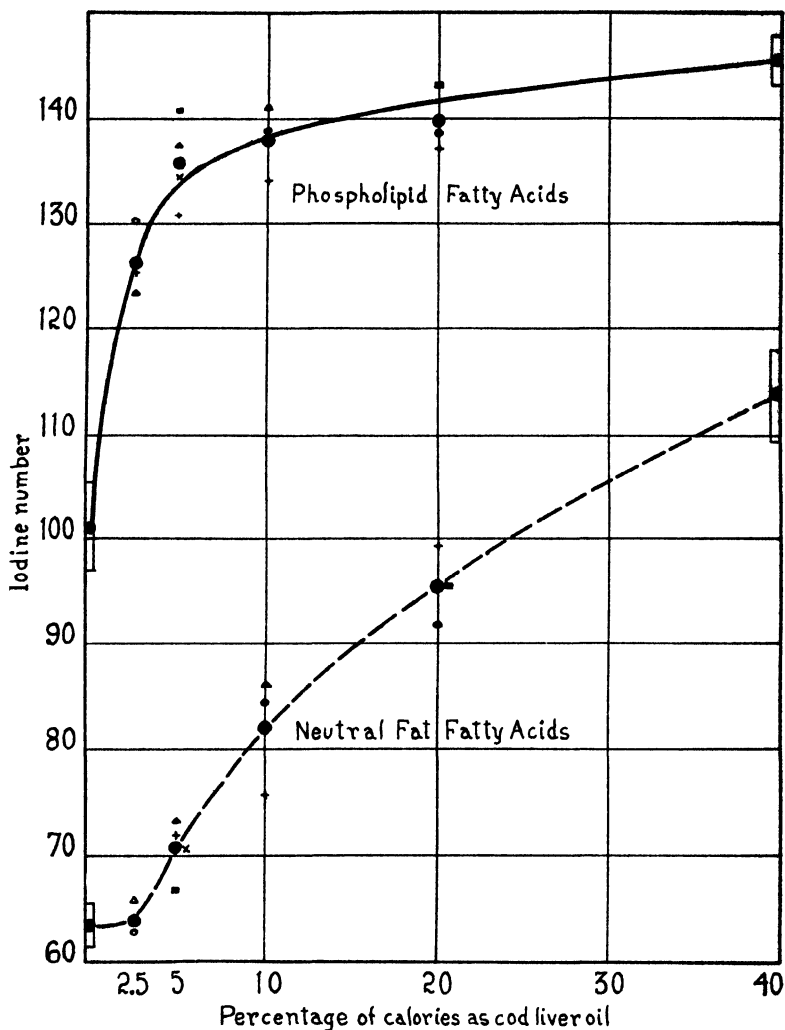


FIG. 2. Showing the relationship between the iodine numbers of the phospholipid and neutral fat fatty acids in the entire bodies of rats and the percentage of the calories provided by cod liver oil. The weights of the individual rats in each group when killed fell between the following limits: +, 69 to 78 gm.; X, 104 gm.; O, 122 to 128 gm.; ■, 142 to 146 gm.; △, 156 to 166 gm.; ● represents averages for the three or four rats which received the same amount of oil each day.

younger rats, and a naturally greater activity and therefore food intake of some rats than of others.

**C. Relationship between Daily Fat Intake and Degree of Unsaturation of Phospholipids in Liver and Skeletal Muscle**—In the two previous experiments data were obtained only on the iodine number of the phospholipid and neutral fat fatty acids in the entire bodies of rats. It was decided to see whether the same relationship between the daily intake of cod liver oil and the degree of unsaturation of the phospholipids as was shown for the entire body of the rat could also be demonstrated to hold for such single organs as the liver and muscles.

The experiments described in an earlier paper (Sinclair, 1931) had by this time shown that the presence in the otherwise fat-free diet of the 0.6 per cent of fat present in the untreated casein and yeast did not have any significant effect on the degree of unsaturation of either the phospholipids or the neutral fat in the tissues of the rat. Consequently, the practice of extracting the casein with hot alcohol and the dried yeast with ether was discontinued. In this present experiment the rats were fed *ad libitum* on our unpurified Diet 3.<sup>6</sup> In addition, each rat received daily by mouth a standard number of drops of cod liver oil delivered from a calibrated dropping pipette. The daily dose has been translated into mg. by taking the average of several weighings of the given number of drops. For the most part the daily dose of oil was given at one time. The two largest doses of 15 and 21 drops were divided until the rats had grown large enough to consume the whole at one time. All rats were kept on the diet until growth had practically ceased.

The results are given in Fig. 3, the iodine number being plotted against the daily dose of cod liver oil. On comparing the two upper curves, it is evident that the effects of small amounts of food fat on the phospholipids of the liver and skeletal muscles are quite different. It may be seen that the ingestion by a rat of just 1 drop of cod liver oil each day is sufficient to raise the iodine number of the phospholipid fatty acids in the liver from

<sup>6</sup> Each rat receiving less than 6 drops of cod liver oil was given daily by mouth the unsaponifiable matter from 150 mg. of cod liver oil dissolved in 2 drops of liquid petrolatum.

the value of about 120, characteristic of the unpurified diet, to as high as 155, while the ingestion of only about 160 mg. is sufficient to raise the iodine number almost if not quite to the maximum.

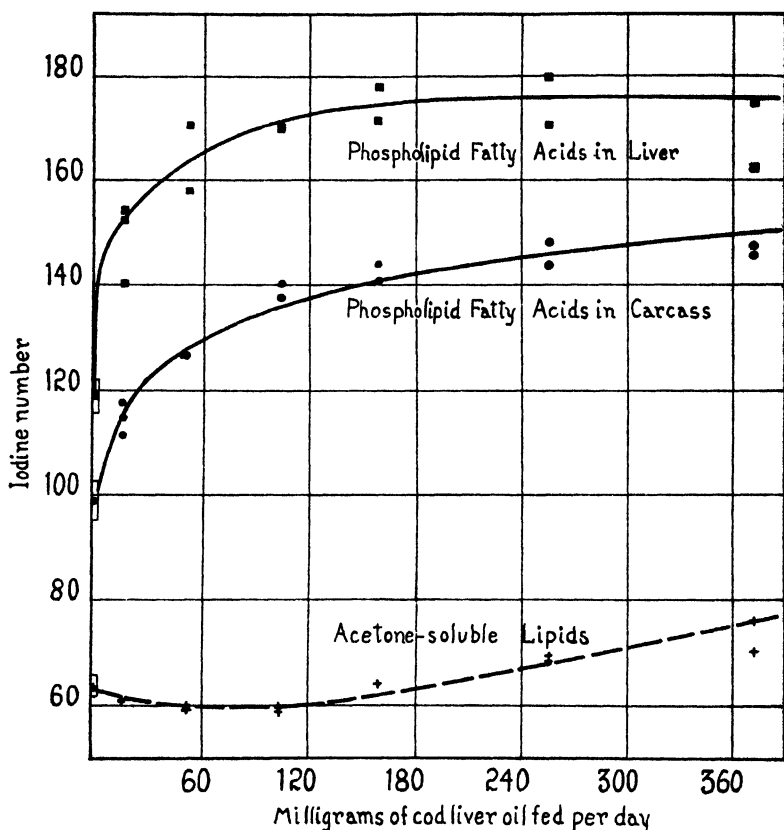


FIG. 3. Showing the relationship between the iodine numbers of the phospholipid fatty acids in the livers and carcasses, and of the acetone-soluble lipids in the carcasses of rats and the amount of cod liver oil ingested each day. ■, phospholipid fatty acids of liver; ●, phospholipid fatty acids of carcass; +, acetone-soluble lipids of carcass.

In the muscles, on the other hand, the relationship between the amount of cod liver oil fed per day and the resultant iodine number of the phospholipid fatty acids is the same as was found true

of the entire body of the rat. The center curve in Fig. 3 is the expression of the formula

$$\text{Iodine No.} = 25.37 \log_{10} \text{amount of cod liver oil} + 84.8$$

This formula was derived from the data by the method of least squares.

It is noteworthy that there is a much closer agreement between the iodine numbers of the phospholipid fatty acids in the muscles than in the livers of rats raised on the same diet. This evidence of greater variability of the composition of the liver phospholipids fits in with the evidence of their more rapid turnover as presented in a recent paper (Sinclair, 1932).

The lowest curve in Fig. 3, showing the absence of any effect of the ingestion of amounts of cod liver oil less than about 100 mg. per day on the degree of unsaturation of the depot fat, is in complete agreement with the results of our first experiment given in Fig. 1.

*D. Comparative Effect of Various Amounts of Coconut Oil and of Lard on Degree of Unsaturation of Tissue Lipids*—In Paper III of this series (Sinclair, 1931) data were presented which showed that the addition of one of a number of fats to the unpurified fat-poor basic ration, in such an amount that 40 per cent of the calories is derived from the fat, results in iodine numbers of the phospholipid fatty acids which are characteristic of the particular fat fed. With coconut oil, for example, the average iodine numbers of the phospholipid fatty acids in the entire animal, in the carcass, and in the liver are 114, 124, and 143, respectively, whereas the corresponding values when cod liver oil is fed are 146, 160, and 184, respectively. In view of the quantitative relationship shown to exist between the iodine number of the phospholipid fatty acids in the muscles of the rat and the amount of cod liver oil ingested each day, the question arose as to whether the influence exerted by all fats on the degree of unsaturation of the phospholipids might not be due to the presence of some common constituent, the concentration of which differs from one fat to another, being high in cod liver oil and low in coconut oil. If such is the case, all fats should tend to elevate the degree of unsaturation of the tissue phospholipids towards the one common maximum. Furthermore, since the iodine numbers indicate that 40



per cent of the total calories as coconut oil is equivalent to 1 per cent as cod liver oil in so far as the tissue phospholipids are concerned, it could be concluded that cod liver oil is about 40 times as rich as coconut oil in the active principle or principles. It seemed reasonable to suppose that if such is the case, since

TABLE I  
*Influence of Various Amounts of Coconut Oil on Degree of Unsaturation of Tissue Lipids*

Amount of coconut oil in diet*	Age	Sex	Weight	Iodine No. of		
				Phospholipid fatty acids in carcass	Acetone-soluble lipids in carcass	Phospholipid fatty acids in liver
	<i>days</i>		<i>gm.</i>			
0.85 per cent by weight, 2 per cent of calories	73	♂	172	100	59	122
78 mg. per day, fed by mouth with dropper	154	♂	250	105	61	143
	317	♂	192	95	62	126
9.4 per cent by weight, 20 per cent of calories	119	♀	160	118	49	149
	140	♂	232	111	51	137
	143	♀	152	114	52	137
19.3 or 21.3 per cent by weight, 37 or 40 per cent of calories	43	♀	80	120	41	
	59	♀	138	122	43	
	129	♀	156	132	44	
	99	♂	222	123	40	144
51.2 per cent by weight, 74 per cent of calories	119	♀	130	126	36	153
	143	♂	264	117	36	139
	145	♀	132	122	38	145

\* All diets were made up so that 18 per cent of the calories came from casein (unpurified), 8 per cent from unextracted dry yeast, and the remainder from cane-sugar and coconut oil. 1 gm. of salt mixture was added for every 100 calories. Those rats which were fed the coconut oil by mouth with a dropping pipette were fed on our unpurified Diet 3.

doubling the amount of cod liver oil has a very marked effect when the concentration is low (Fig. 2), then doubling the intake of coconut oil should also produce a pronounced increase in the iodine number of the phospholipid fatty acids. To test this point rats were raised on diets in which 2, 20, 40, and 74 per cent of the total calories were derived from coconut oil. The casein

provided 18 per cent of the calories in all cases. Each rat received daily the unsaponifiable matter from 150 mg. of cod liver oil dissolved in 2 drops of liquid petrolatum. The results obtained are given in Table I. Although not conclusive, the data indicate that the feeding of increasing amounts of coconut oil tends to elevate the degree of unsaturation of the phospholipids, not to the same level as cod liver oil, but to a level which is characteristic of coconut oil.

In conjunction with the experiments described in Section B, three rats were raised on a diet which contained only 1.1 per cent of lard; that is, 2.5 per cent of the calories. The phospholipid fatty acids in the entire bodies of these rats had iodine numbers of 116, 117, and 114. This increase of about 15 over the iodine number characteristic of the basic fat-free ration amounts to 60 per cent of the average increase when the lard provides 40 per cent of the calories.

#### DISCUSSION

*Is There a Causal Relationship between Low Degree of Unsaturation of Phospholipids and Subnormal Growth of Rats Raised on Fat-Free Diets?*—At the time that the author embarked upon the study of the influence of the amount and character of the food fat on the composition of the phospholipids in the tissues of the rat, little information was available as to whether or not rats would grow on a diet which was practically devoid of fat. Soon afterwards the papers by McAmis, Anderson, and Mendel (1929) and by Burr and Burr (1929) appeared. These authors presented evidence to show that the total exclusion of neutral fat from the diet, even if all of the known vitamins are supplied in presumably adequate amounts, is incompatible with normal growth and well being. In so far as growth is concerned, the observations of the author agree with such a conclusion.

Without exception the rats which were raised on either our fat-free Diet 20 or our unpurified Diet 3 (the latter contained an average of 0.64 gm. of fatty acids per 100 gm. of ration) grew more slowly and to a smaller maximum weight than did those rats which were raised on similar diets containing some added fat. Representative growth curves are given in Fig. 4. Curve 1 is the composite growth curve of three litter mate male rats which

were raised on the unpurified fat-poor diet and which received daily by mouth the unsaponifiable matter from 150 mg. of cod liver oil dissolved in 2 drops of liquid petrolatum. Some of the rats grew somewhat larger than these three, but the curve is a fair illustration of the growth of male rats on this diet. Curve 2 is the composite growth curve of ten male rats on our unpurified

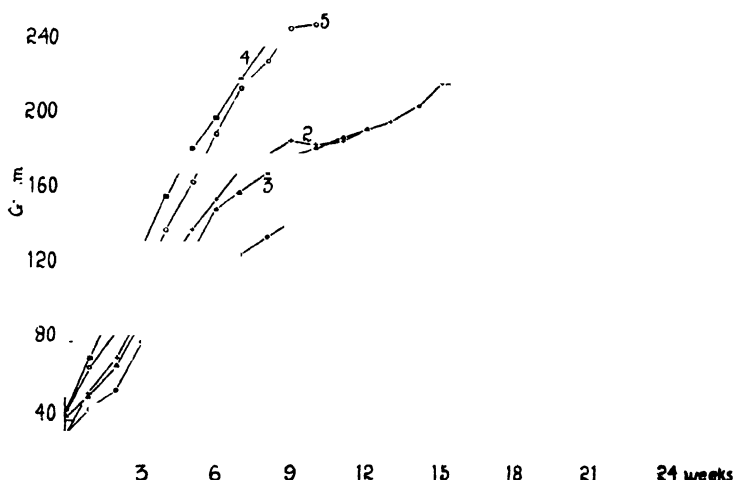


FIG. 4. Comparative growth curves of rats raised on various diets. Curve 1, composite growth curve of three male rats raised on our unpurified Diet 3 supplemented daily with the unsaponifiable matter from 150 mg. of cod liver oil dissolved in 2 drops of liquid petrolatum. Curve 2, composite of ten male rats raised on unpurified Diet 1 which contained 87 mg. of oscodal per 100 gm. of ration. Curve 3, composite of six male rats raised on Diet 3 supplemented daily with the unsaponifiable matter from 150 mg. of cod liver oil dissolved in 78 mg. of coconut oil. Curve 4, growth curve of a male rat raised on Diet 262 containing 21.3 per cent of coconut oil. Curve 5, growth curve of a male rat (litter mate of that in Curve 4) raised on Diet 262 containing 21.3 per cent of linseed oil. Each 100 gm. of both coconut oil and linseed oil contained the unsaponifiable matter from 5 gm. of cod liver oil.

Diet 1, in which oscodal was mixed with the dry ration. Curve 3 is the composite growth curve of six rats which were raised on the unpurified diet supplemented daily by the unsaponifiable matter from 150 mg. of cod liver oil dissolved in 78 mg. of coconut

oil. Curves 4 and 5 illustrate the growth of rats on diets which were similar to the unpurified fat-poor diet except that 40 per cent of the total calories, which in the latter were derived from cane-sugar, were in the former supplied by coconut oil and linseed oil, respectively. On comparing Curves 1 and 2 with Curves 4 and 5, it is evident that the addition of fat even to our unpurified diet (which is poor in fat but not fat-free) exerts a markedly beneficial effect on growth.

The question arises: is the poor growth of rats raised on the fat-free or fat-poor diets due to the abnormally low degree of unsaturation of the tissue phospholipids, and is the beneficial effect of food fat on growth the result of the elevation in the level of unsaturation of the phospholipids? Essentially the same question has already been asked and answered in the affirmative by Burr and Burr (1930). On the basis of their observation that the various symptoms of what they termed "a new deficiency disease produced by the rigid exclusion of fat from the diet" could be prevented and cured only by the feeding of linoleic acid, Burr and Burr proposed the hypothesis that "warm blooded animals in general cannot synthesize appreciable quantities of linoleic acid and some of the other highly unsaturated acids . . . [which] are normal constituents of essential cellular phospholipids. . . ."

In an earlier paper the author (1930,*b*) has called attention to the fact that scaliness of the feet and tail, apparently identical with the condition described by Burr and Burr (1929) as one of the early symptoms of the fat deficiency disease, may develop in rats which are receiving cod liver oil in the diet. It was also stated that while these peculiar lesions were invariably observed on the tails of rats kept in false bottom cages and fed on the fat-free diet, none of the symptoms of the fat deficiency disease with the exception of subnormal growth was observed when the rats on exactly the same diet were raised in the ordinary stock cages. It was suggested that the rats were protected by some factor which they obtained by consuming their feces. Recently Burr, Burr, and Brown (1931) have confirmed our observation that cod liver oil does not prevent the development of the tail lesions in rats raised in metabolism cages. Within the past year three different groups of workers have described a scaly condition of the tail which is similar to that described by Burr and Burr (1929) and yet is

neither prevented nor cured by the feeding of unsaturated fats (Graham and Griffith, 1931; Funk, Caspe, and Caspe, 1931; Hume and Smith, 1931). There seems to be scarcely any doubt that whatever may be their etiology, the peculiar lesions which appear on the tails of rats raised on a fat-free diet are not connected in any way with the degree of unsaturation of the tissue phospholipids.

However, the above question as to the possibility of a causal relationship between subnormal growth and the low degree of unsaturation of the phospholipids still stands. In spite of numerous attempts the author has not yet succeeded in securing normal growth of rats on diets which at the same time will leave the tissue phospholipids with the same low level of unsaturation that is found in rats raised on the fat-free or fat-poor diets. The most successful was that illustrated by Curve 3 in Fig. 4, in which the unsaponifiable matter from cod liver oil, to supply vitamins A and D, was fed daily by mouth in about 78 mg. of coconut oil. While this small amount of coconut oil had no significant effect on the degree of unsaturation of the phospholipids, there was a very marked improvement in growth over that illustrated in Curve 1. It is noteworthy that these rats which received 78 mg. of coconut oil daily grew to an average maximum weight of 228 gm., but after about 6 months they began to lose weight. The improvement in growth which resulted when the vitamins A and D were given in coconut oil instead of in liquid petrolatum, as in Diet 3, together with the better growth of the rats on Diet 1, which contained a comparatively large amount of oscodal mixed in the dry ration, has served to strengthen the author's conviction that the deficient growth on fat-free diets is due in part at least to a deficient utilization of the essential fat-soluble vitamins. Another contributory cause of the subnormal growth of our own rats on the fat-poor diets undoubtedly was the high incidence of snuffles, resulting no doubt from the partial deficiency of vitamin A (Boynton and Bradford, 1931).

In spite of the fact that one could maintain that part of the beneficial effect of fat ingestion on the growth of rats is due to the improvement in the utilization of vitamin A (and possibly of other unknown accessory factors) with the consequent increase in resistance to infections and in general well being, nevertheless

there seems to be at the present time no evidence which precludes the possibility that fat in the diet exerts a beneficial effect by virtue of its ability to increase the degree of unsaturation of the phospholipids. However, this latter explanation of the beneficial effect of food fat on growth should be regarded as no more than a mere possibility until some definite information has been obtained as to the function of the tissue phospholipids and the purpose of their content of unsaturated fatty acids. While admitting the possibility that the subnormal growth of rats on diets which contain negligible amounts of the more highly unsaturated fatty acids is the result of an insufficiently high level of unsaturation in the tissue phospholipids, we are able to conclude that there is certainly no parallelism between either the rate of growth or the general well being of rats and the degree of unsaturation of their tissue phospholipids. For example, Curves 4 and 5 in Fig. 4 show that growth on a diet containing 20 per cent of coconut oil may be even slightly better than on one containing linseed oil, although the latter yields phospholipid fatty acids with much the higher iodine number. Also, the general behavior and rate of growth of rats raised on coconut oil and cod liver oil do not differ in any apparent respect. Frequently, indeed, the growth on cod liver oil is rather inferior to that on various other fats; this may be due to the presence in cod liver oil of toxic substances (Norris and Church, 1930).

*Synthesis of Fatty Acids by the Rat*—One of the original purposes of this investigation was to determine the degree of unsaturation and eventually the composition of the phospholipids in the tissues of rats which had been compelled to synthesize all of their fatty acids. It was hoped that in this way information would be obtained as to what factors other than diet exert an influence on the composition of the phospholipids. It was soon realized, however, that because of the immense difficulties involved in obtaining active concentrates of the various vitamins, an *absolutely* fat-free diet was not for the present attainable. Our nearest approach to a fat-free diet still contained about 0.25 gm. of fatty material per 100 gm. of mixed ration, most of the fat being present in the extracted yeast. In view of the very marked influence which small amounts of cod liver oil are now known to exert on the degree of unsaturation of the phospholipids, it is obviously impossible

to say to what extent the fat present in the extracted yeast has been involved in the building up of phospholipid fatty acids with the mean iodine number of about 100 which, as we have seen, is quite characteristic of the basic fat-free ration. There is, however, some reason for believing that other factors inherent in the tissues of the rat are responsible for the uniformity of this level of unsaturation, and that the fat present in the basic fat-free ration has played little or no part in the synthesis of phospholipid. This belief is based on the fact that the same level of unsaturation is observed in rats raised on the unpurified diet which contains 2.5 times as much fat as the fat-free diet. On the basis of the results obtained with added cod liver oil, it is reasonable to believe that had the small amount of residual fat in the basic ration exerted any influence on the phospholipids, an increase of 2.5 times would have caused a very pronounced increase in the degree of unsaturation. The average iodine number of about 100 is probably characteristic of synthetic fatty acids when they are built into the phospholipid molecule, just as about 60 is their characteristic iodine number when they are deposited as neutral fat in the depots. One may well ask what are the inherent factors which are responsible for the maintenance of these two distinct levels of unsaturation in the fatty acids which are synthesized and built up into phospholipid and neutral fat by rats raised on a fat-free diet? For the present this question apparently must remain unanswered.

Mention has already been made of the hypothesis put forward by Burr and Burr (1930) that the rat is unable to synthesize linoleic acid, and presumably the other highly unsaturated fatty acids which are essential constituents of the tissue phospholipids. The observations of the author, although not sufficiently definite to deny its truth, are quite opposed to such a hypothesis. It is evident that even though the rate of growth and the maximum weight attained are subnormal, rats will grow on a fat-free diet. With growth there must be synthesis of new phospholipid. If, according to the above hypothesis, rats are unable to synthesize the more highly unsaturated acids, when the diet is devoid of fat, they must be compelled to make their new phospholipid out of oleic and the two common saturated acids, stearic and palmitic. Consequently the maximum iodine number which the new phos-

pholipid fatty acids can have is that of oleic acid (90). Since, so far as is known at present, the mixed phospholipids always contain some saturated acids, the actual iodine number must fall considerably below 90. Presumably (according to the hypothesis) when this takes place, the phospholipids do not satisfactorily fulfil their essential functions.

Actually, as shown in this and a previous paper (Sinclair, 1931), the iodine numbers of the phospholipid fatty acids in the tissues of rats raised on either a fat-free or an unpurified fat-poor diet are very seldom as low as 90 and usually approximate 100. Low as it is, this mean iodine number of 100 for a mixture of fatty acids is undeniable proof of the existence of appreciable quantities of the more highly unsaturated fatty acids. When it is remembered too that the values given throughout this work are about 10 per cent too low because of the presence of contaminating lipid material of low degree of unsaturation (Sinclair, 1931), and that the phospholipid fatty acids always contain some saturated acids, it is evident that the amount of the highly unsaturated acids must be quite considerable. In the light of the facts revealed by the study of the rate of phospholipid metabolism (Sinclair, 1932), the question arises as to whether these highly unsaturated acids may not be those which were originally present when the rat was placed on the fat-free diet. There are at least two reasons for believing that such is not the case. First, the phospholipid fatty acids in the body of a rat which had been on the fat-free diet for 11 days and had increased in weight from 32 to 50 gm. gave an iodine number of 99; if the original 400 mg. of fatty acids with a mean iodine number of 124 had been retained, the 215 mg. of new phospholipid fatty acids must have had a mean iodine number of 6. Secondly, it has been found that the iodine number of the phospholipid fatty acids does not decrease progressively during growth but decreases rapidly from the initial value of 124 to approximately 100, at which level it apparently remains over a long period of time. At this point it must be stated that there is some evidence that if the rats are kept for a period of several months on the fat-free or unpurified diet, the phospholipid fatty acids may have iodine numbers considerably below 100. For instance, the phospholipid fatty acids in the entire bodies of four rats which had been on the unpurified diet for about 9 months had the unusually



low iodine number of 81.<sup>7</sup> In this case, however, the low value was due partly to a rather high percentage of the contaminating material mentioned above. It is certainly not impossible that the premature decline and death of rats on the fat-free diet as observed by Burr and Burr (1929) are associated with the drop in the degree of unsaturation of the phospholipids below a certain level which is essential for their function.

The presence of fatty acids of as high a degree of unsaturation as arachidonic in the mixed phospholipid fatty acids even when the mean iodine number is as low as 81 has been definitely proved by brominating the liquid acids after separating into solid and liquid fractions by the lead salt-alcohol method. Despite the fact that a considerable amount of liquid acids separated out with the solid fraction, the yield of ether-insoluble bromides amounted to 6.9 gm. per 100 gm. of the total phospholipid fatty acids. In another experiment, in this case on the phospholipid fatty acids from the carcass, the yield of ether-insoluble bromides was 4.0 gm. per 100 gm. of fatty acids. Whether or not these highly unsaturated fatty acids originated in the small amount of yeast and casein fat present in the fat-free and unpurified diets can of course only be decided by further work. To the author it seems more reasonable to believe that they were synthesized by the rat, and that the animal organism in general is able to synthesize all of the various fatty acids which are usually found in animal tissues. Otherwise it is difficult to account for the presence of arachidonic acid, and also of the 24-carbon cerebroside acids, in the tissues of the Herbivora.

*Influence of Ingested Fatty Acids on Degree of Unsaturation of Tissue Phospholipids*—Paper III of this series (Sinclair, 1931) showed the importance of the character of the food fat as a factor governing the degree of unsaturation of the tissue phospholipids. Added to this information we now have, in the results of this present study, evidence of the preeminent importance of the presence of small amounts of fat in the diet. We have seen: (1) that the daily administration of only 1 drop (17 mg.) of cod liver

<sup>7</sup> Recently the phospholipid fatty acids in the carcass and liver of a rat which had been fed on the unpurified diet for 339 days and which weighed 206 gm. when killed, were found to have iodine numbers of 90 and 114, respectively.

oil to a rat which is fed on the unpurified diet increases the iodine number of the phospholipid fatty acids in the liver and carcass by 29 and 16 per cent, respectively; (2) that the daily ingestion of only about 150 mg. of cod liver oil by a rat increases the degree of unsaturation of the phospholipids of the liver almost to the maximum ever observed; (3) that there is a definite quantitative relationship between the amount of cod liver oil fed per day and the iodine number of the phospholipid fatty acids in the entire animal and in the skeletal muscles, irrespective of the body weight of the animal; (4) that comparatively small amounts of lard and, to a less extent of coconut oil, also exert a marked influence on the degree of unsaturation of the tissue phospholipids; (5) that the small amounts of fat which exert such marked effects on the level of unsaturation of the phospholipids have no demonstrable effect on that of the depot fat, the latter apparently being entirely of synthetic origin.

The unmistakable difference in the effect of small amounts of food fat on the phospholipids of the liver and skeletal muscle, together with observations made in previous work, leads to the conclusion that the various organs of the body may be divided into at least two groups with respect to the metabolism of their phospholipids. In the one group are the intestinal mucosa, the liver, and the blood, in all of which there is a very rapid turnover in their constituent phospholipids within a few hours after the ingestion of a characteristic fat such as cod liver oil; in the other group are the skeletal muscles and presumably the other organs of the body, in which the rate of phospholipid turnover is much less rapid than in the former group. The rapid turnover of the phospholipids in the intestinal mucosa was interpreted as evidence of their involvement as an intermediary stage in the resynthesis of absorbed fatty acids into neutral fat (Sinclair, 1929); the rapid turnover of the phospholipids in the liver<sup>\*</sup> (Sinclair, 1929, 1932) and in the blood (unpublished experiments) requires much further study before it can be satisfactorily explained. As pointed out in the preceding paper (Sinclair, 1932), the func-

<sup>\*</sup> The astonishingly great effect of small amounts of ingested cod liver oil on the degree of unsaturation of the liver phospholipids might be interpreted as the result of the absorption of fat directly into the portal circulation.

tion of the phospholipids in the skeletal muscles, which undergo under certain conditions rather a rapid turnover, is also quite obscure; it is believed that their function is not as an intermediary product in the metabolism of fat.

It is necessary to bear in mind that two quite distinct facts have been brought out by this and preceding studies of the influence of food fat on the composition of the phospholipids of such tissues as the skeletal muscles; first, that the amount and character of the food fat exert a decisive influence on the degree of unsaturation of the *new* phospholipid continuously being synthesized by the growing rat; secondly, that the degree of unsaturation of the *preformed* phospholipid in the tissues of the full grown rat will shift rapidly from a lower level to a higher in response to an appropriate change in the character of the food fat (Sinclair, 1932). This present discussion concerns only the probable explanation of the very definite quantitative relationship which exists between the food fat and the type of phospholipid synthesized by the growing rat.

As we have seen from the results presented in this paper and in the two preceding papers in the series, if the rat is raised on a diet which contains only such fat as is present in the casein and dried yeast, the constituent fatty acids of the newly formed phospholipids in the entire animal and in the skeletal muscles have a mean iodine number of about 100. Since removal of much of the fat from the casein and yeast does not decrease the level of unsaturation of the phospholipids, it seems probable that the unsaturated acids which have been used are mainly, if not entirely, of synthetic origin. If, however, the rat receives a certain constant amount of fat (such as cod liver oil) in addition to the basic ration, the iodine number of the phospholipid fatty acids is increased above 100, the extent of the increase being a function of the character and the amount of the extra fat fed.

The results of our studies have shown that cod liver oil occupies an outstanding position among the various fats which have been fed, with respect to the level of unsaturation induced in the tissue phospholipids. It is also known that cod liver oil differs markedly from these various other fats (linseed oil, lard, olive oil, butter, and coconut oil), not so much in the average degree of unsaturation of its fatty acids, as in having a high content of the more highly

unsaturated fatty acids such as arachidonic acid. Consequently, it seems to be a reasonable conclusion that the ingestion of highly unsaturated acids results in a higher than normal percentage of the same unsaturated acids in the tissue phospholipids. Furthermore, since the phospholipid fatty acids, with a few rare exceptions (of which those in the tissues of rats fed on linseed oil are an example), are always considerably more unsaturated than the neutral fat fatty acids, the question has arisen as to whether or not the phospholipids constantly strive to attain, and to maintain, a certain optimum level of unsaturation which, in the skeletal muscles, is indicated by an iodine number at least as high as 160. The results of this present study indicate that such is not the case. In the first place, it will be remembered that the iodine numbers obtained when various fats are fed do not trend towards the same common level but rather towards a certain maximum which is characteristic of each particular fat. This maximum iodine number is above 160 for cod liver oil, about 140 for linseed oil and lard, slightly lower for olive oil, and around 125 for coconut oil and butter. In the second place, the iodine numbers of the phospholipid fatty acids in the entire animal and in the muscles tend to remain at a constant level, depending upon the amount of oil fed per day, irrespective of the rate of growth and of the body weight. If there were a tendency for the iodine number to approach an optimum level somewhat above 160, such a tendency should be revealed by a progressive increase in the iodine number as the rate of growth and, therefore, the rate of phospholipid synthesis become less and less.

It is the author's belief that, although much more work remains to be done, the most satisfactory explanation at the present time for the observed relationship between both the nature and the amount of the fat in the diet and the degree of unsaturation of the phospholipids in such tissue as the skeletal muscles, is that the composition of the phospholipids synthesized by the growing rat is dependent upon at least these two factors: first, the relative rates at which the various fatty acids, both saturated and unsaturated, react with the phosphoric acid-base complex to form phospholipid; secondly, the relative concentration of the various fatty acids within the centers of phospholipid synthesis.

## SUMMARY

In this paper a study is made of the relationship between the amount of fat (usually cod liver oil) ingested by the rat and the degree of unsaturation of the phospholipid and neutral fat fatty acids in the entire animal, the carcass (*i.e.* chiefly the skeletal muscle), and the liver.

It is shown that:

1. The feeding of small amounts of cod liver oil to rats on an otherwise "fat-free" or fat-poor diet results in an astonishingly great increase in the iodine number of the phospholipid fatty acids over the low value characteristic of the basic ration.

2. There is a definite quantitative relationship between the amount of cod liver oil ingested per day, and the iodine number of the phospholipid fatty acids irrespective of the rate of growth or body weight of the animal.

3. Small amounts of fat have a much more pronounced effect on the phospholipids in the liver than on those in the skeletal muscles.

4. The feeding of small amounts of other fats than cod liver oil, such as lard and, to a less extent, coconut oil, also causes a marked increase in the degree of unsaturation of the tissue phospholipids.

5. The small amounts of fat which are sufficient to produce a marked increase in the level of unsaturation of the phospholipids have no apparent effect on the degree of unsaturation of the neutral fat.

The possibility of a causal relationship between the low degree of unsaturation of the tissue phospholipids and the subnormal growth of rats on fat-free diets, the question of the synthesis of fatty acids by the rat, and the factors governing the composition of the tissue phospholipids, are discussed.

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## THE ANTINEURITIC VITAMIN

### II. REMOVAL OF IMPURITIES BY OXIDIZING AGENTS\*

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In a previous paper on this subject (1), a simple method was described for preparing a salt-free concentrate of vitamin B<sub>1</sub> by increasing the alcohol concentration from 50 to 100 per cent. The alcoholic filtrate contained large amounts of carbohydrate gums in addition to the vitamin and nitrogenous impurities. It was believed that if these gums could be oxidized to small acidic molecules without a loss in potency, the process by which this was brought about would be of value in further work aimed at concentration and isolation of the antineuritic substance.

Zilva (2) found that autolyzed yeast filtrate exposed to ozone for 6 hours lost very little of its potency. Sherman and Smith (3) referring to the work of Kinnersley and Peters say that at an acidic reaction, in general, the active material proved to be stable to oxidizing agents. These results combined with the indirect evidence of the stability of the antineuritic vitamin to oxidation as indicated by its resistance to nitrous acid (4) led us to study the conditions necessary to achieve our object.

Three general methods of oxidation were carried out: (a) oxidation of the salt-free concentrate (1) at 100° for 10 to 20 hours, (b) oxidation of the salt-free concentrate at 61° (in presence of constant boiling mixture of alcohol-water-carbon tetrachloride) for 5 to 10 hours, and (c) oxidation of the impure fullers' earth concentrate at 61° from 3 to 48 hours. Since the third method

\* This work was aided by a grant from the Chemical Foundation to the Department of Biological Chemistry, and by the Research Fund at Yale University School of Medicine.



was the best, not only in ease of operation but also in results obtained, it alone will be described in detail.

A vitamin concentrate prepared for us by Eli Lilly and Company from 1000 pounds of rice polishing was used as the starting material. A small aliquot of this material containing 30 gm. of total solid was acidified with concentrated hydrochloric acid. To this solution an excess of barium chloride and 2 volumes of ethyl alcohol and carbon tetrachloride respectively were added. The solution was warmed to a gentle boil and a suitable quantity of the oxidizing agent added. The reaction was allowed to proceed in an apparatus essentially the same as described in "Organic syntheses" (1, 5) except that the mechanical stirrer was omitted and a dropping funnel substituted in its place. The reaction was allowed to proceed until no more water distilled over. The salts were removed by filtration and the solvents and excess acid were removed by concentration *in vacuo*. The product was tested by the method of Block, Cowgill, and Klotz<sup>1</sup> (1).

The variations employed in this technique were (a) type of oxidizing agent, (b) ratio of amount of oxidizing agent to organic solids, (c) sequence of addition of oxidizing agent, and (d) time of heating.

*Oxidation with Nitric Acid*—As it was impossible to calculate the amount of oxidant necessary to destroy the complex carbohydrates in the vitamin solution without injuring the potency, we were forced to proceed on a purely empirical basis. It was thought that the best criterion would be the ratio of the number of gm. of total organic solids in the solution to the number of cc. of concentrated nitric acid used. Eight experiments were carried out, differing only in the organic solids-nitric acid ratios. It was found that under the above conditions the vitamin was completely destroyed by nitric acid at 61° for 7 hours, if the ratio was below 1.5; ratios of 1.5 to 3.0 resulted in partial destruction, and above 3.2 the vitamin activity of the solution was unchanged (Table I). Further experiments indicated that if the ratio of the organic solids to the nitric acid was greater than 3.2, neither the order of adding the oxidant (before or after the alcohol) nor the time of heating (5 to 48 hours) influenced the results. The vitamin solutions

<sup>1</sup> The results reported in this paper required approximately 100 individual tests averaging 2 weeks each.

obtained were clear pale yellow. Unfortunately, in spite of repeated concentration *in vacuo*, the vitamin solutions contained traces of oxides of nitrogen which made them undesirable.

*Oxidation with Sodium p-Toluenesulfochloramide*—The amount of chloramine-T added in these experiments was based on the total organic solids of the vitamin solution. The results obtained with this oxidant showed that it is a very efficient oxidizing agent toward the carbohydrate gums present; the amount of nitrogen and the vitamin potency remain practically unchanged even when the ratio of the number of gm. of oxidant to the number of gm. of organic solids is 0.3 (Table I). However, use of this reagent required the introduction of an extra step in order to remove the toluene derivatives formed by the reaction, and for this reason this type of oxidation is undesirable.

*Oxidation with Inorganic Substances*—Several attempts were made to use chromic anhydride and potassium permanganate; it was found, however, that these substances yielded reduction products which were not easily removed from the vitamin solution, and for this reason their use cannot be recommended. It is of interest to report that these oxidants, in small amounts, did not destroy the vitamin activity (Table I).

*Oxidation with Hydrogen Peroxide*—This substance from a theoretical as well as a practical point of view proved to be the ideal oxidant. Seven experiments were carried out with various amounts of 30 per cent hydrogen peroxide. The ratios were calculated by dividing the total number of gm. of organic solids by the number of cc. of hydrogen peroxide used. It was found that ratios of 0.2 to 0.4 resulted in a partial destruction (20 to 40 per cent) of the vitamin, while ratios above 0.5 yielded a clear reddish yellow transparent vitamin solution of undiminished potency (Table I). This material was practically salt-free. When assayed by the method of Block, Cowgill, and Klotz (1), it proved to be potent in amounts of less than 8 mg. of total solids per pigeon unit. Further evidence that this concentrate contained the antineuritic factor was demonstrated by tests for "anorexia-correcting" power and ability to relieve typical polyneuritic symptoms in vitamin B-deficient dogs<sup>2</sup> and pigeons. In

<sup>2</sup> We are indebted to Dr. E. Burack for the tests on dogs. These experiments will be described in detail elsewhere.

these tests the material was administered both parenterally and by mouth.

*Preparation of Large Amount of Vitamin B<sub>1</sub> Concentrate by Oxidation-Carbon Tetrachloride Technique*—Rice polishings were extracted (Eli Lilly and Company) with dilute hydrochloric acid, and the vitamin adsorbed on fullers' earth at pH 4.5. The fullers'

TABLE I  
*Effect of Various Oxidizing Agents on Antineuritic Vitamin*

Experiment No.	Oxidising agent	Ratio of total solids in vitamin solution to cc. or gm. of oxidant	Recovery of vitamin	Degree of concentration based on total solids
			per cent	
1	Fuming HNO <sub>3</sub>	3.5	100	3 ×
3	" "	3.2	100	3 ×
5	" "	2.0	30	2 ×
7	" "	1.2	0	
9	" "	0.6	0	
11	Cr <sub>2</sub> O <sub>3</sub>	5.0	100	0 ×
13	"	1.0	20	-5 ×
15	"	0.5	0	
17	KMnO <sub>4</sub>	2.0	100	-3 ×
19	"	0.5	0	
21	Chloramine-T	1.0	100	1 ×
23	"	0.5	60	-2 ×
25	"	0.4	75	-2 ×
27	"	0.3	75	1 ×
29	H <sub>2</sub> O <sub>2</sub>	0.5	100	2 ×
31	"	0.4	95	2 ×
33	"	0.3	65	1 ×

The experiments described in this table were always carried out in duplicate. The oxidizing agent was added before the alcohol in the one experiment and after in the other.

earth was washed with water, and the vitamin eluted by sodium hydroxide at about pH 13. The alkaline solution was immediately brought to pH 2 by hydrochloric acid and the solution concentrated to a syrup. This black, sticky gum was diluted until the total solid concentration was about 30 per cent and purified as follows: To 1 liter of the vitamin solution the following reagents were added; 200 cc. of concentrated hydrochloric acid, 150 cc. of

a 10 per cent barium chloride<sup>3</sup> solution, 1500 cc. of 95 per cent ethyl alcohol, and 1500 cc. of technical carbon tetrachloride. The contents were well mixed and brought to a gentle boil on the steam bath in the apparatus previously described (1). To this warm solution, 125 cc. of 30 per cent hydrogen peroxide<sup>4</sup> (Merck) were carefully added. The solution became rapidly lighter in color, and after heating for 1 hour it was a clear yellow. The boiling was continued until all the water was removed (absolute alcohol and carbon tetrachloride were added as necessary). As a result of the long heating, the solution gradually became darker; this color was removed by adding 2 to 3 cc. of 30 per cent hydrogen peroxide at the end of the reaction and heating 15 minutes longer. The suspension was allowed to stand at 5° overnight. The insoluble salts were filtered off, washed with alcohol-carbon tetrachloride, and the filtrate concentrated to dryness *in vacuo*. The precipitate was dissolved in water and again evaporated to dryness. This was repeated twice and the residue dissolved (melted) in a minimal amount of boiling water. This suspension was allowed to stand at 5° overnight, filtered, washed, and diluted to a known volume. The solution contained all of the original vitamin potency. It was free from inorganic salts, yielded no amino nitrogen in the Van Slyke apparatus, and was suitable for parenteral injection. There was no appreciable decrease in total nitrogen during this procedure. This concentrate can serve as a starting point for further work on the chemistry of the vitamin as well as a source of the "pure" antineuritic vitamin.

#### SUMMARY

A simple modification of the carbon tetrachloride technique (1) which involves a concurrent oxidation of carbohydrate impurities without appreciable loss of antineuritic vitamin is described.

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<sup>3</sup> Barium chloride is added until no further precipitate appears. This is essential; otherwise toxic substances are formed by the reaction.

<sup>4</sup> Chlorine was not detected during this reaction.

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## MUCOPROTEIN AS A NORMAL CONSTITUENT OF THE GASTRIC JUICE\*

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In the glands of the gastric mucosa are cells which in their histological characteristics differ from the typical mucin-producing cells but which have staining reactions somewhat similar to those of the known mucin-secreting cells (Zimmermann, 1925). The presumption was made that the product of their secretion is a mucin-like substance and a normal constituent of the gastric juice (Babkin, 1928, 1931; Webster, 1931).

It was found by one of us (W., reported by Babkin (1929)) that a substance could be precipitated from the filtered and neutralized gastric juice by acetic acid, which gave a considerable reduction after hydrolysis with dilute mineral acids and contained no phosphorus. Another preparation was obtained by acidifying the filtered and neutralized gastric juice with acetic acid and adding 1 volume of acetone. This substance also gave a strong reduction after hydrolysis. From this substance a body could be prepared having the properties of a sodium salt of mucoitin sulfuric acid (Webster, 1930).

There are three possible explanations for the presence in the pure gastric juice of a substance having the properties of a mucoprotein or its derivatives. (1) It might be secreted as such from the mucoid cells of the gastric glands. (2) Mucus secreted by the surface epithelium might be dissolved in the gastric juice. (3) This latter may be first digested and the products of digestion dissolved. This possibility was suggested by the work of Pekelharing (1902) who observed that the gastric mucus when washed with water and alcohol is not soluble in hydrochloric acid of a

\* A preliminary communication appeared in *Am. J. Physiol.*, **97**, 569 (1931).

concentration corresponding to the normal acidity of the gastric juice, but on digestion with pepsin and hydrochloric acid gradually loses this characteristic and dissolves partially, leaving a sediment easily soluble in alkali.

This investigation was undertaken in order to study the properties of the protein constituents of the gastric juice, with a view to establishing their relation to the mucoproteins. An attempt was made to eliminate the digestion of the mucus secreted by the surface epithelium.

### *Methods*

Six dogs with esophagotomy and gastric fistula were used for the collection of the juice.

*Analytical Methods*—Estimation of carbon and hydrogen was performed by the combustion method. Nitrogen was determined by Dumas' method.

Total sulfur was determined by Carius' method.

Organic sulfates were determined as  $\text{BaSO}_4$  after heating with 3 per cent hydrochloric acid for 10 hours in a sealed tube in a boiling water bath.

For phosphorus determinations the moist ashing procedure ( $\text{H}_2\text{SO}_4 + \text{HNO}_3$ ) was used and the molybdate test performed. As this was always negative, the procedure of Fiske and Subbarow (1925) was also used as a control.

Reducing power was estimated after hydrolysis in 2 N  $\text{H}_2\text{SO}_4$  according to the method of Hagedorn and Jensen (1923).

### *Experimental Results*

The analytical data to be discussed here were obtained chiefly on three protein preparations which were isolated from the gastric juice collected after sham feeding.

*Preparation I*—This preparation was obtained from gastric juice which was collected as follows: The stomach of the dog was washed out four times with 300 cc. of warm tap water and once with distilled water. Portions of juice were collected on sham feeding every quarter to half hour and each was at once filtered through a separate filter and immediately neutralized with NaOH and precipitated with acetic acid, added up to 1 per cent, and 1½ volumes of acetone. A pure white flocculent precipitate appeared

immediately. After standing overnight in a refrigerator the clear liquid was decanted. The precipitate was freed from the mother solution by centrifuging and twice washed with acetone water (1.5:1). The resulting substance, when carefully washed with alcohol in increasing concentration (till chlorine-free) and ether, dried in a vacuum desiccator, and finely powdered, was a white powder with a faint grayish shade, very slightly soluble in distilled water, insoluble in dilute acetic and mineral acids, and insoluble in concentrated acetic acid. It gave general protein color reactions such as the biuret, xanthoproteic, Millon, Molisch, Liebermann, Adamkiewicz, and the lead acetate test. It did not reduce Fehling's or Benedict's solutions without previous hydrolysis, but gave a strong reduction after 3 hours hydrolysis in 2 N sulfuric acid. Its reducing power, determined in four estimations, was found to correspond to 12.6 to 12.8 per cent glucose.

The substance did not give an orcinol test according to Bial's (1903) modification, nor Seliwanoff's (1887) resorcinol test. Phosphorus was not found. On analysis the following results were obtained.

N	13.82, 14.02 per cent
S	0.8 per cent
Ash	3.85 " "

The ash was clearly soluble in distilled water, giving a neutral solution. Sodium, iron, and sulfates were found to be present in the ash. Chlorine was not detected.

The presence of organically combined sulfates could be demonstrated.

The high reducing power after hydrolysis in the absence of phosphorus, low nitrogen content, and presence of organic sulfate indicated that we were dealing with a substance having some of the properties of a mucoprotein. It was very peculiar that in spite of extensive washing the preparation still contained a considerable amount of ash. Since the ash was completely soluble in distilled water and the solution was neutral and contained sulfate but did not contain chlorine, there was little doubt that sodium was combined with radicals of sulfuric acid of the carbohydrate compound.

It was of interest to investigate in what form the protein body



in question exists in the freshly secreted acid gastric juice; whether as a salt or as free acid.

In the course of investigation it was found that it is possible to precipitate practically the whole complex of protein bodies from the gastric juice directly by 2 volumes of acetone. It was therefore decided to use this procedure in attempts to elucidate further the composition of the proteins of the gastric juice.

*Preparations II and III*—These two preparations were obtained by direct precipitation with acetone from samples of gastric juice collected from two dogs under different conditions of stimulation; Preparation II by continuous feeding and Preparation III by feeding for 5 minutes at intervals of 1 hour.

The aim was to obtain some information as to how the intensity of nervous impulses and the individuality of the animal would reflect on the amount and composition of the protein complex secreted. Collection of the juice was performed in both cases over a period of 3 hours; samples were taken every 15 to 30 minutes, filtered separately, and precipitated directly by 2 volumes of acetone. The precipitate appeared and settled down rapidly. It was thus quite possible to decant the supernatant liquid after 30 minutes, but since it was decided to perform the whole analysis as far as possible quantitatively, the whole sample was first collected and the protein precipitates were then separated. In the case of Preparation II isolated from 2 liters of gastric juice this could be accomplished in 2 weeks; Preparation III was obtained from the juice collected during 4 weeks, but separation of proteins was performed twice from 2 liter portions taken at 2 weeks intervals.

Both Preparations II and III were treated as follows: Absolutely clear supernatant liquid was removed by syphoning. The combined precipitates, together with the balance of the mother liquid, were centrifuged, washed four times with acetone water, 2:1, several times with 75 to 80 per cent alcohol until chlorine-free, then with alcohol of increasing concentration from 80 per cent to absolute, and finally washed with absolute ether, and desiccated *in vacuo* over sulfuric acid in the presence of soda-lime. The precipitates obtained were perfectly white in appearance immediately after precipitation and during washing, but when dried they turned gray at first, gradually assuming a reddish

<sup>1</sup> By acetic acid alone or combined with acetone.

Since the data of analysis and the general properties of Preparations II and III were practically identical, the conclusion may be drawn that the protein complex of gastric juice is the same in different animals and under varying conditions of stimulation. There was great similarity in general properties and analytical data between Preparations II and III and Preparation I, isolated by acetone precipitation after neutralization with NaOH and acidulation with acetic acid. The nitrogen, carbon, and hydrogen values and the reducing power were identical, but a striking feature of Preparation I was the presence of ash in considerable amount. This is probably due to the formation of sodium salt, resulting from the use of NaOH in the process of isolation. The last two preparations were practically ash-free, indicating that in the acid gastric juice protein molecules are not combined with metal ions as salt, and do not form any stable complex compounds with inorganic salts.

#### DISCUSSION

Levene (1925) and his coworkers have brought forward a mass of experimental evidence to prove that mucoproteins are built up as are nucleoproteins from a simple protein and a characteristic prosthetic group, this group being a conjugated sulfuric acid; namely, chondroitin or mucoitin sulfuric acid.

In our investigation there was no opportunity to secure adequate quantities of gastric juice to enable us to isolate and identify this prosthetic group. Therefore we decided to limit ourselves to the task of obtaining some indirect evidence of the possible presence of the mucoprotein in the gastric juice. The following characteristics would be presumptive evidence that the substance is a mucoprotein: (1) low nitrogen content; (2) high reducing power after hydrolysis with dilute mineral acids in the absence of organic phosphorus; (3) presence of organically combined sulfates.

The comparatively low nitrogen percentage, the presence of organic sulfates in the isolated substances, the considerable reducing power after hydrolysis with dilute mineral acid (which, estimated in different ways, varies from 12.8 to 15 per cent), and the negative determinations for phosphorus and pentoses—all these may be satisfactorily explained only by the presence in the preparations investigated of some compound belonging to the group

of mucoproteins. The analytical data obtained are not quite typical as compared with classic representatives of mucoproteins, such as mucin from the submaxillary glands, since the nitrogen (12.32 per cent) in those is considerably lower and the reducing power higher. Reduction in the class of mucoproteins, however, varies widely; *e.g.*, submaxillary mucin 20 per cent, tracheal secretion 30 to 35 per cent, mucoprotein from ovarian cysts 10 per cent, blood serum mucoid 25 per cent. The reduction power of our preparations lies therefore within the limits observed in the group of mucoproteins. On the other hand, our preparations have a distinctly higher reducing power than has ever been observed in other phosphorus-free proteins. The highest value reported for albumin is 10 to 11 per cent (Seemann, Fehling, Knapp, Langstein). Osborne and Campbell and Mörner and Spenser, however, failed altogether to obtain a positive reduction test on ovalbumins (quoted after Levene (1925)). According to Levene the presence of a carbohydrate radical in the molecules of the group of albumins is a debated question. Probably this is due to admixture of the mucoid (Seemann).

On comparison with some of the pepsin preparations obtained by Pekelharing (1896-97, 1902) from pure gastric juice, our preparations showed some degree of similarity in analytical data. Nitrogen values obtained by Pekelharing for different preparations varied from 14.13 to 14.75 per cent. His higher nitrogen figures were observed in preparations isolated by precipitation with ammonium sulfate and were probably due to the contamination by ammonium salts; but his lower values are fairly close to ours. Similarly, his carbon and hydrogen values are also very close to those of our preparations, being for C from 51.61 to 52.32 per cent and for H from 6.93 to 7.19 per cent. Such deviations, if still present, can be satisfactorily explained when one takes into consideration that Pekelharing did not wash his preparations sufficiently (once with a small amount of distilled water). Some very suggestive analytical data concerning the composition of highly purified pepsin have been reported by Fenger *et al.* (1927, 1928). Their preparations, which were obtained from the mother solutions after the greater part of the highly active pepsin had been removed, gave a nitrogen value of 13.33 per cent; in the first acetone precipitate, where mucin is said to be present, the nitrogen value was

13.52 per cent, whereas in the pepsin precipitate, obtained at pH 2.5 to 3.8, the nitrogen was 14.20 per cent. Our preparations also resemble in many respects that of López-Suárez (1913), obtained by him from the mucus removed from the gastric wall. On drying, a gray-white powder was formed, which was clearly soluble in alkali and insoluble in dilute mineral acids. On acid hydrolysis a strongly reducing fluid was obtained and much organic sulfate was split off. In his analysis the following percentages were obtained: C 49.63, H 7, N 13.48, S 1.75, P 0.77, ash 0.75. López-Suárez claims it was in no way possible to obtain the substance free from phosphorus and from substances which on hydrolysis liberated purine bases.

Davis and Merker (1919) obtained from a commercial pepsin a very active preparation of pepsin. Their work is of interest in connection with the present investigation because they regarded pepsin as a glucoprotein. They were led to this conclusion by certain properties found by them in the preparation. It gave a positive Molisch test and did not contain chlorine, but contained 0.47 per cent  $P_2O_5$ , 2.01 per cent ash, and 1.01 per cent CaO. The N content was 13.77 per cent, S 1.5 per cent. The high content of P and ash, and especially of calcium, indicates that this preparation was probably to a very great extent contaminated with products of digestion of the elements of the gastric mucosa, particularly with the surface mucus. However, the conception of pepsin as a glucoprotein is not in agreement with the recent investigations of Northrop (1929-30). He obtained highly active preparations of pepsin in crystalline form, which gave a negative result with the Molisch test. Therefore, it seems more probable that pepsin, though, as Northrop states, itself a protein, is secreted by the gastric glands in combination with a glucoprotein and possibly with other proteins. Nothing definite is known about the mutual relations of different proteins in the gastric juice.

An attempt was made to estimate roughly, at least, the composition and general properties of the mucous secretion of the surface epithelium of the gastric mucosa. On the basis of certain physiological evidence we assumed that only threads and lumps of mucus, which constitute a greater part of alkaline gastric secretion in the fasting condition, may be considered with some degree of certainty as products of the activity of the mucous cells of the

gastric surface epithelium. Therefore the procedure was adopted which conveniently permitted the separation of this substance in a fairly pure state. It was found that, if to the alkaline gastric secretion an equal volume of alcohol be added, the lumps and threads of mucus within a few minutes float on the surface of the liquid, while the small amount of flocculent precipitate which is formed simultaneously settles down. It was thus possible mechanically to separate the threads and lumps of mucus. In this way the surface epithelium mucus was obtained from 90 cc. of alkaline secretion in a dog with gastric fistula and esophagotomy.

This substance was rapidly rinsed several times with 50 per cent alcohol in a beaker, washed several times alternately with distilled water and 0.1 N hydrochloric acid, washed free of chlorine with alcohol (in increasing concentration from 50 to 80 per cent), then three times with absolute alcohol, once with dry acetone, and twice with dry ether. The product obtained was desiccated to constant weight in a vacuum desiccator over sulfuric acid in the presence of soda-lime. The yield calculated to the original material was 0.6 per cent. The resulting product was pure white, very light, and not as hygroscopic as the preparations previously described. At room temperature it was soluble in water to 0.5 per cent, insoluble in dilute acetic acid and mineral acids, and readily soluble in alkali. From alkaline solutions, on acidifying with acetic or hydrochloric acid, a viscous precipitate was formed which tended to adhere to the stirring rod in the manner characteristic of typical mucins (*e.g.* submaxillary mucus). All general color protein reactions were positive. There was no reduction before hydrolysis, but after hydrolysis with 2 N  $\text{H}_2\text{SO}_4$  for 3 hours in the boiling water bath the resulting liquid gave a strong reduction with Fehling's and Benedict's solutions. In these experiments the reduction was found to correspond to 31, 33, and 35 per cent glucose. The substance contained 2 per cent ash and 12.26 per cent nitrogen or 12.51 per cent when calculated for ash-free substance. The material contained organic sulfates.

These data may be considered sufficient to allow us to come to the conclusion that the product isolated from the surface mucus belongs to the typical mucins, like that of the tracheal secretion or submaxillary mucin. The gastric surface epithelium mucus, however, differs greatly in every respect, even in the outward

appearance of the purified dry substance, from the preparations obtained from the filtered acid gastric juice.

## SUMMARY

1. We believe sufficient data have been advanced to establish the presence in the freshly secreted gastric juice of a protein exhibiting the general properties of a mucoprotein.

2. The striking similarity in the elementary composition of different samples obtained from several animals and its variance from that of the mucus of the surface epithelium point to this being a *sui generis* chemical body, secreted by the cells of the gastric glands themselves.

The writers wish to express their thanks to Dr. B. P. Babkin who directed this work.

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# VITAL NEED OF THE BODY FOR CERTAIN UNSATURATED FATTY ACIDS

## I. EXPERIMENTS WITH FAT-FREE DIETS IN WHICH SUCROSE FURNISHES THE SOLE SOURCE OF ENERGY\*

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Until recently, the chief physiological rôle assigned to dietary fats has been that which considered them merely as fuel for the expenditure of energy. In fact, the idea has prevailed that so long as the demands for the fat-soluble and other vitamins were satisfied, animals could dispense with fats entirely<sup>1</sup> (1-3).

When, in 1927, Evans and Burr (4) attempting further purity of synthetic diets, employed sucrose as the sole source of energy, the foundations were laid for the recognition of two phenomena, both of which deal with the rôle of fats in the physiological economy of the rat. One of these phenomena deals with a peculiar relationship of fats to vitamin B which they spare; that is, in the presence of fats, less vitamin B is required than in their absence. This was reported by Evans and Lepkovsky (5) in 1928. The other phenomenon deals with the impossibility of normal growth or physiological well being in rats on diets adequate in all known respects, but rendered rigidly fat-free. This was reported simultaneously in 1929 by Burr and Burr (6) and McAmis, Anderson, and Mendel (7). The deficiency of rats on fat-free diets is characterized by impairment of growth after an initial growth had been accomplished, dermatitis which results in scaliness of the skin and tail,

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<sup>1</sup> In 1921, Osborne and Mendel presented a review of the literature on the indispensability of fats in diets (2).



an abnormally large intake of water, which is lost chiefly through pathways other than the kidney, and kidney lesions causing the appearance of blood in the urine. Burr and Burr carried the investigation further (8) by showing definitely that linoleic acid and possibly other unsaturated fatty acids cure this deficiency.

An explanation for the failure to elicit the deficiency when diets contain corn-starch became evident when it was found in this laboratory that the trace of fatty acids in the starch, about 0.5 per cent, was sufficient when fed to afflicted rats on fat-free diets to cure them in a spectacular manner. Taylor and Iddles (9) have shown that without previous hydrolysis of corn-starch, appropriate solvents cannot remove all of its fatty acids. It is for this reason that the mere extraction of corn-starch with acidulated alcohol does not decrease its effectiveness as a cure for the new malady. The corn-starch fatty acids include linoleic acid (10) and our disclosures are accordingly in harmony with Burr and Burr's demonstration of the efficacy of pure linoleic acid in relieving this disorder.

When corn-starch is hydrolyzed to the erythrodextrin stage, the dextrin can be precipitated with alcohol; the liberated fatty acids remain in solution in the alcohol. The dextrin is no longer effective as a cure for the disorder produced in fat-free diets, whereas the fatty acids are quite effective.

Potato starch, which contains phosphoric acid and no fatty acids (9), is ineffective as a cure of the malady produced on fat-free diets.

Glycogen, by the very nature of its preparation, is bound to be fat-free, and is ineffective when fed to animals which receive no unsaturated fatty acids.

Rice starch which contains double bonded fatty acids is effective. The fatty acids from the rice starch are also effective.

Unfortunately, some of the symptoms associated with the deficiency disease on fat-free diets, such as scaliness and tail necrosis (6, 8), are not specific for this deficiency. Both scaliness (11-13) and tail necrosis (14) in rats have been described as occurring with diets containing an abundance of fats. Indeed, tail necrosis almost identical with that described by Burr and Burr (6) was reported by Parsons (11) when the diet contained curative fatty acids such as linoleic acid and curative fats such as linseed

oil. On the other hand, out of hundreds of rats on fat-free diets in our laboratory not a single clear cut case of tail necrosis has appeared.

The situation is complicated by the observations of Graham and Griffith (15), and confirmed by Burr, Burr, and Brown (16), that cod liver oil will not cure the condition of scaliness. However, the latter writers claim that growth is definitely stimulated. Nor is the situation simplified by the observations of Sinclair (17) that when rats have access to their feces, scaliness does not develop, though growth is just as seriously retarded. This is disputed by Burr, Burr, and Brown (16) who report that two rats after having access to their feces for 5 weeks showed no very marked improvement in either scaliness or growth.

The relation of accessibility to feces to the occurrence of scaliness has recently been reviewed by Hume and Smith (18). In fact, they use the "scaly tail" as the important symptom in their investigations. They suggest that coprophagy is a factor insufficiently controlled, and is the cause of such varying results as are recorded in the literature. They are inclined to look upon deficiency in vitamin B as the most probable cause of "scaly tail." They support this contention with the evidence that scaly tail is more easily produced on large wire mesh screens than on small wire mesh. It seems clear to them from this that coprophagy has augmented the vitamin B intake. As further evidence they show that addition of unautoclaved yeast alleviates the condition. They suggest that the work of Evans and Lepkovsky on the sparing<sup>2</sup> action of fat on vitamin B may provide the explanation of the cause of scaly tail.

We cannot explain the disorder produced by rigidly fat-free diets as due to inadequate vitamin B. We have conducted crucial experiments here, employing several times the adequate amount of vitamin B. Furthermore, amounts of fats which are effective in curing the peculiar new deficiency disease on fat-free diets, are quite ineffective in any sparing effect on vitamin B. Thus the mere addition of 2 drops of cod liver oil daily prevents for a long

<sup>2</sup> They also look upon fat as having a sparing action on vitamin G, and doubt the claims of Evans and Lepkovsky that fat has no sparing action on vitamin G. Shortly, we intend to publish additional data on the non-sparing effect of fat on vitamin G which it is hoped will be more convincing.

time<sup>3</sup> the appearance of symptoms characteristic for a fat-free diet but does not markedly spare vitamin B.

Unfortunately, Hume and Smith employ rice starch in their diets. It is known that rice starch carries fatty impurities, and since these are very potent in relieving distress in rats on fat-free diets, it is not logical to expect the characteristic symptoms obtained on fat-free diets to develop with rats on this diet.

All of our work on rigidly fat-free diets amply confirms the essential facts laid bare by Burr and Burr (6, 8), and McAmis, Anderson, and Mendel (7). We are also able to confirm the additional work by Burr and Burr on the essential rôle played by unsaturated fatty acids. The essential facts, we consider, are the cessation of growth of rats on the fat-free diet, emaciation and obvious malnutrition, and the response of these animals to small amounts of certain fatty materials. We would also lay great stress on increased water intake and on hematuria. While scaliness or dandruff is also almost always observed, we do not stress the importance of this symptom, and as for tail necrosis, we have been obliged to ignore it as a symptom, since we have not observed it.

#### EXPERIMENTAL

*Preparations of Fractions Fed*—A starch fat preparation was made from the fatty material obtained as a by-product in the manufacture of corn syrup.<sup>4</sup> The material as we received it was extracted with ether; the ether was then evaporated and the residue taken up in pentane. 1 cc. of the pentane solution carried 0.33 gm. of starch fat (labeled Supplement 36-c).

The solution of fatty acids of rice starch was prepared by autoclaving for about 40 minutes a 10 per cent solution of rice starch in 0.01 per cent HCl. After cooling, the hydrolyzed starch was extracted with ether and the resulting solution was such that 1 cc. was equivalent to 7 gm. of rice starch.

The "pure" oleic acid used was prepared from commercial oleic

<sup>3</sup> Animals receiving a sucrose-casein diet which has not been treated with fat solvents and which is supplemented with 2 drops of cod liver oil daily exhibit symptoms similar to those described after animals had been on the diet for a year or more.

<sup>4</sup> This was kindly furnished us by the Corn Products Company of Argo, Illinois.

acid. The solid acids were removed by Twitchell's lead soap-alcohol method (19). The liquid acids were then converted into the barium soap, and thrice recrystallized from equal volumes of dry benzene and absolute alcohol after the method of Skellon (20).

The linoleic acid was only about 90 per cent pure and was isolated from corn oil by conversion of the oil to methyl esters, distilling them, and then fractionating the liberated fatty acids by Twitchell's (19) and Farnsteiner's (21) methods.

The liquid fatty acids from coconut oil were prepared from the residue which remained in the flask of our fractionating column (22) after the removal of most of the saturated fatty acids. This residue consisted of some palmitic acid and most of the liquid fatty acids of coconut oil. The liquid fatty acids were separated from the palmitic acid by Twitchell's (19) method. Although the liquid fatty acids consisted mostly of oleic acid, there was some linoleic acid present, as shown by the presence of bromides insoluble in pentane.

The dextrin was prepared from corn-starch by autoclaving for 15 minutes a 10 per cent starch solution in 0.001 per cent HCl. Under these conditions the starch was carried to the erythro-dextrin stage, very little reducing substances having been formed by this mild hydrolysis. The dextrin was precipitated with 95 per cent ethyl alcohol, and the alcohol then removed.

The corn-starch and potato starch were extracted with 95 per cent ethyl alcohol containing dry HCl gas according to the method of Taylor and Nelson (23).

Two samples of glycogen were prepared, one by the method of Pflüger (24), and the other by Sahyun and Alsberg's method (25).

*Preparation of Animals*—The basic diet was essentially the same as that used in the early work of Evans and Burr (4). The supplements, however, were treated with fat solvents and were similar to those used by Burr and Burr (6).

*Diet 616*

Casein (Van Slyke).....	24.0
Sucrose.....	72.1
Salt Mixture 185 (26).....	3.9

Diet 616 was supplemented with (1) 1.0 gm. of ether-extracted Northwestern yeast, (2) the non-saponifiable matter of cod liver

oil (27) equivalent to 83 mg. daily, (3) the non-saponifiable matter of wheat germ oil equivalent to about 500 mg. weekly.<sup>5</sup>

The animals were placed on this diet on the 21st day of life and maintained three in a cage on raised wire screens. The growth and the occurrence of estrum were observed. Up to the 60th to 70th day the growth was practically normal, as was also the incidence of estrum. From this time on, growth was not only retarded but gradually reached a plateau, accompanied by fewer incidences of estrum. The age at which the cure materials were started differed with various groups. The average age, however, was about 135 days although occasional animals did not exhibit the deficiency until they attained 180-200 days. This marked resistance of some animals to the deficiency is not explained. Recovery of well being and increase in weight were our criteria for determining the potency of the fractions fed. Scaliness, while noted, was not an important criterion in our work, since the animals were not always fed curative doses long enough for it to disappear entirely. The clearing up of hematuria was also a valuable guide in our work.

### *Results*

The results obtained from feeding the various substances are most easily appreciated by inspection of Figs. 1 to 9. The curves given in this paper represent the 30 day period just previous to the administration of the material being tested, which is indicated in all cases by a heavy line. The circles interrupting the curve in each case represent the occurrence of estrum. The letter *H* indicates the presence of hematuria.

Because of the relation of vitamin B to fat we thought it necessary to determine the effect of adding excessive quantities of this vitamin to the fat-free diet (Diet 616). Fig. 1 shows that exces-

<sup>5</sup> The wheat germ oil is prepared by a cold percolation of wheat germ in glass containers with benzene and the removal of the benzene in a vacuum. The oil is saponified by adding 400 gm. of wheat germ oil to a boiling solution consisting of 160 gm. of KOH, 100 cc. of water, and 700 cc. of 95 per cent alcohol, and boiling for 2 minutes. About 3 liters of water are added and the mixture is cooled under running water. After cooling, it is extracted four times with ether, the total volume generally being about 2.5 liters, which is finally concentrated to 200 cc.

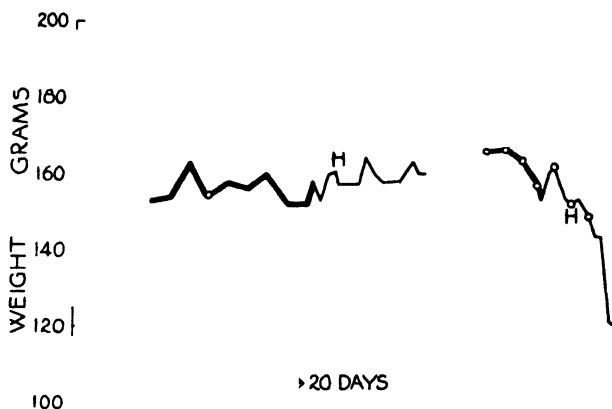


FIG. 1. Body weights of animals which were reared and maintained on the casein-sugar diet (Diet 616 plus supplements) and then given excessive amounts of vitamin B. *H* indicates hematuria.

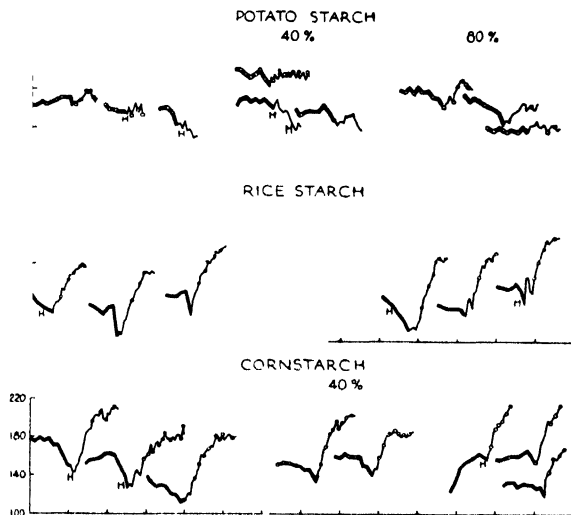


FIG. 2. Body weights of animals which were reared and maintained on the casein-sugar diet (Diet 616 plus supplements) and then given diets in which various amounts of potato starch, rice starch, and corn-starch replaced equal amounts of the sugar. *H* indicates hematuria.

sively large amounts of vitamin B do not materially improve the nutritive deficiency which develops with fat-free diets. In the case of these two animals, the amount of vitamin B was increased by supplementing the diet with 3 cc. of a 25 per cent alcoholic extract of rice bran (1 cc. = 4 gm. of rice bran), bringing the vitamin B intake to at least 8 times the necessary amount.

Fig. 2 shows the results obtained when the diet contained varying amounts of either potato starch, rice starch, or corn-starch. Very prompt relief of the state of malnutrition was obtained with the rice starch and corn-starch, whereas no marked response was obtained with the potato starch. Three levels of corn-starch were fed and when the diet contained as little as 20 per cent there was a marked response. Two levels of rice starch were fed—50 and 80 per cent of the diet—with a marked response in both cases. Diets containing 20, 50, and 80 per cent potato starch were fed but no marked responses were obtained.

The composition of the diets fed to this group is as follows:

Diet No.	Casein (Van Slyke)	Sugar	Corn-starch	Rice starch	Potato starch	Salt Mixture 185 (26)
	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>
618	16.0		80.1			4.0
618-A	16.0	40.1	40.1			3.9
618-B	16.0	60.1	20.0			3.9
618-G	16.0				80.1	3.9
618-H	16.0	40.1			40.1	3.9
618-I	16.0	60.1			20.1	3.9
668-A	16.0	30.1		50.0		4.0
668-B	16.0			80.1		3.9

Fig. 3 demonstrates the fact that the further extraction of corn-starch with acidulated alcohol does not decrease its effectiveness in alleviating the deficiency, whereas corn-starch freed of its fatty acids and fed as dextrin produces no response.

Fig. 4 shows that fatty acids from corn-starch (Supplement 36-c) are effective in supplying the deficiency on these highly purified fat-free diets.

Fig. 5 shows that the fatty acids from rice starch supply the deficient factor when fed to animals on a fat-free diet.

Fig. 6 shows that glycogen has little or no activity. The glyco-

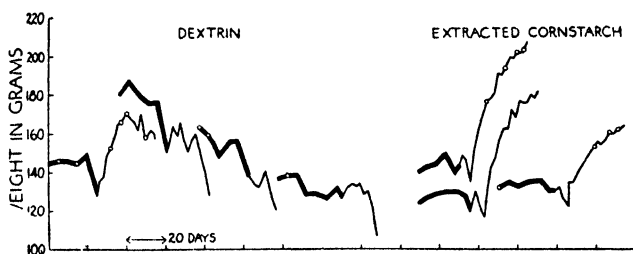


FIG. 3. Body weights of animals which were reared and maintained on the casein-sugar diet (Diet 616 plus supplements) and then given a diet in which 50 per cent of the sugar was replaced by (1) corn-starch which had been extracted with acidulated alcohol and (2) corn-starch freed of its fatty acids (dextrin).

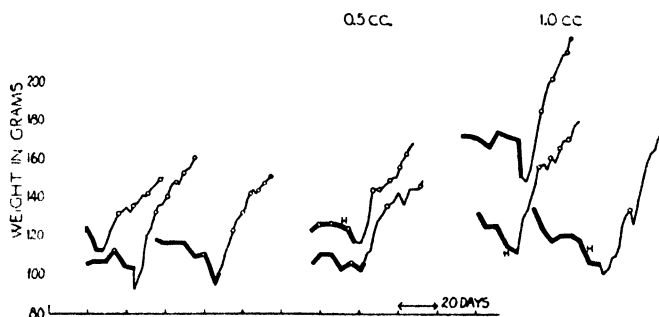


FIG. 4. Body weights of animals which were reared and maintained on the casein-sugar diet (Diet 616 plus supplements) and then fed the fatty acids from corn-starch (Supplement 36-c) at three levels (0.1, 0.5, and 1.0 cc.). *H* indicates hematuria.



Fig. 5. Body weights of animals which were reared and maintained on the casein-sugar diet (Diet 616 plus supplements) and then fed the fatty acids from rice starch at two levels (1 and 3 cc.).



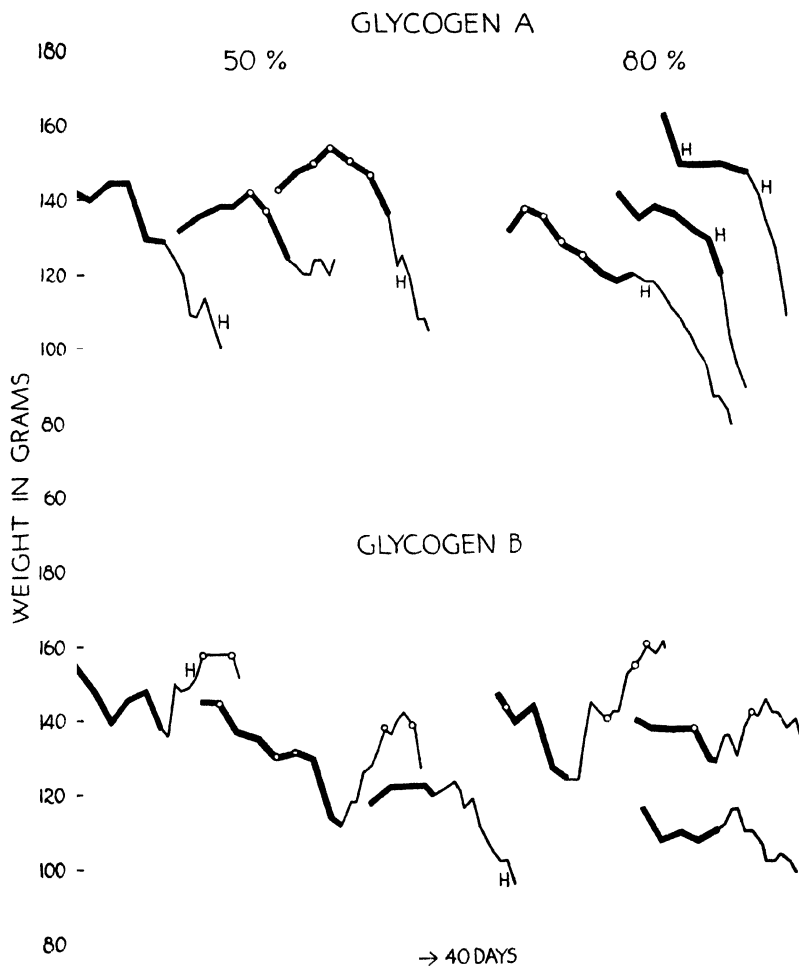


FIG. 6. Body weights of animals which were reared and maintained on the casein-sugar diet (Diet 616 plus supplements) and then given diets in which glycogen replaced 50 or 80 per cent of the sugar. Two types of glycogen were fed, Glycogen A, prepared according to the method of Pfüger, Glycogen B, prepared according to the method of Sahyun and Alsberg. H indicates hematuria.

gen prepared by alkaline hydrolysis (Glycogen A) in accordance with Pflüger's method seems actually to be toxic. Whether this is apparent or actually the case we cannot say with certainty.

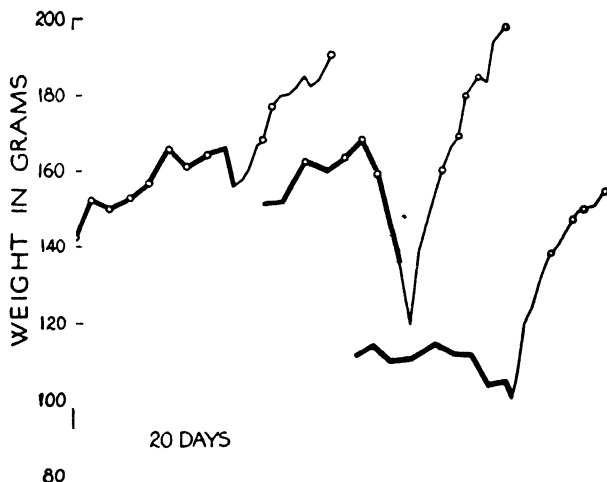


FIG. 7. Body weights of animals reared and maintained on the casein-sugar diet (Diet 616 plus supplements) and then given 5 drops of the liquid acids from coconut oil daily.

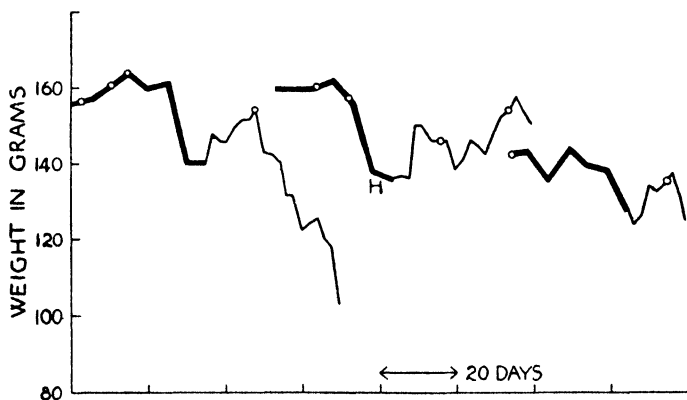


FIG. 8. Body weights of animals reared and maintained on the casein-sugar diet (Diet 616 plus supplements) and then given 5 drops of pure oleic acid daily. *H* indicates hematuria.

The glycogen prepared with trichloroacetic acid (Glycogen B) according to the method of Sahyun and Alsberg does not seem to possess this toxic action.

Fig. 7 shows that the liquid acids from coconut oil are effective. This fact is interesting because Burr and Burr report coconut oil ineffective. However, it is not at all out of harmony with their findings, since the liquid acids of coconut oil contain linoleic acid.

The "pure" oleic acid carefully freed of linoleic acid is quite ineffective (Fig. 8).

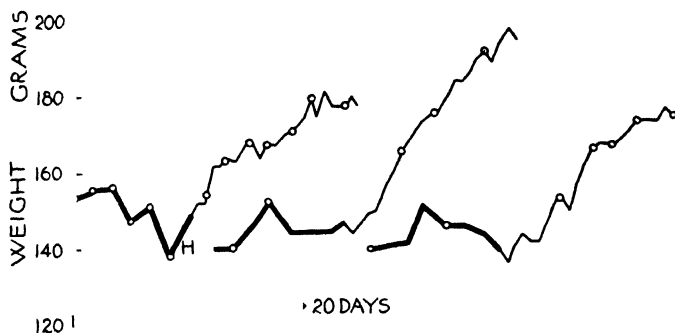


FIG. 9. Body weights of animals reared and maintained on the casein-sugar diet (Diet 616 plus supplements) and then given 5 drops of linoleic acid daily. *H* indicates hematuria.

Fig. 9 shows that linoleic acid (about 90 per cent pure) is very effective.

#### DISCUSSION

There is little room for doubt that certain fatty fractions play a prominent physiological rôle in the rat. The evidence presented so far by Burr and Burr is indicative that only certain fatty acids, namely linoleic and possibly other unsaturated fatty acids, are the active agents.

It seems as though the manifestations characterizing the deficiency are not uniform in all laboratories. As an instance, we have not observed tail necrosis, whereas it seems the usual occurrence, or at least a common occurrence in Burr's laboratory. It is

difficult to classify scaliness as a symptom, or to assign to it definite meaning because it has been so often observed on animals receiving a diet which was not fat-free.

Since most, if not all, of the natural fats contain the requisite traces of the necessary fatty acids, and since corn-starch and rice starch, so commonly used in synthetic diets, also contain these essential fatty acids, it is natural that the phenomenon herein discussed did not have much opportunity of coming to light except on fat-free diets. Yet we wish to mention the fact that the mere presence of fats, even in large amounts, will not prevent this deficiency provided unsaturated fatty acids with more than one double bond are carefully excluded. These results will be discussed in more detail in Paper II of this series; they are mentioned here only to emphasize the fact that the disorder with which we are dealing is not actually produced by a fat-free regimen but only by the exclusion of certain fatty acids normally present in fat.

#### SUMMARY

1. The fact that a specific deficiency disease is produced on rigidly fat-free diets and is cured by the addition of certain unsaturated fatty acids is confirmed.

2. Vitamin B has been definitely ruled out as a limiting factor in this deficiency.

3. Corn-starch and rice starch are effective in curing the deficiency, whereas potato starch is ineffective. The curative carbohydrates owe their effectiveness to their slight but important content in unsaturated fatty acids—presumably linoleic acid.

4. Hog liver glycogen, whether prepared by hydrolysis with alkali or precipitation with trichloroacetic acid, is ineffective.

5. Tail necrosis, so commonly reported, has not been observed in this laboratory.

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## **VITAL NEED OF THE BODY FOR CERTAIN UNSATURATED FATTY ACIDS**

### **II. EXPERIMENTS WITH HIGH FAT DIETS IN WHICH SATURATED FATTY ACIDS FURNISH THE SOLE SOURCE OF ENERGY\***

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In our studies on the physiological importance of fats to the animal organism (1), we became convinced that progress could be made only when individual fatty acids were used. Even the most simple natural fat is a complex entity. In addition to glycerides of fatty acids, we have the substances collectively called the non-saponifiable matter. From the present standpoint these may be looked upon as impurities associated with the glycerides of the fatty acids. The glycerides themselves are complex; witness the multiplicity of differing fatty acids, saturated and unsaturated, present, and the possible isomeric arrangements in the glycerol molecule. Only when synthetic fats composed of single fatty acids are used in experimental diets will it be possible to accumulate a body of data which will lead to a better understanding of the rôle played by fats in the animal organism.

The present communication is concerned with the rôle of single saturated fatty acids when vitamin B and, in fact, all the known vitamins are adequately supplied. When single fatty acids were used as the sole source of energy in the diet, certain phenomena akin to those obtained in studies on fat-free diets were manifested (2, 3). There were discovered similarities, but also some vital differences.

\* Aided by grants from the Committee for Research in Problems of Sex of the National Research Council and from the Rockefeller Foundation. These funds have been generously augmented by the Board of Research and the College of Agriculture of the University of California.

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Even at very high levels, fats have been found to be excellent sources of energy (1). Theoretically, therefore, single fatty acid glycerides should serve equally well as a source of energy. Certainly no reason is known why glycerides of pure fatty acids should not serve as sources of energy as well as sucrose. We therefore fed two basal dietary mixtures to rats, one having sucrose as the sole source of energy and the other, glyceryl laurate. In the latter diet no carbohydrate was fed, the only source of carbohydrate being that contained in the yeast and that which was converted from protein by the rat. The two basal diets have the following composition:

<i>Diet 639</i>		<i>Diet 616</i>	
Glyceryl laurate.....	60.0	Sucrose.....	72.1
Casein.....	40.0	Casein.....	24.0
Salt Mixture 185 (4).....	5.0	Salt Mixture 185.....	3 9

The difference in protein represents an attempt to keep the nutritive ratio as nearly the same as possible.

To our surprise the growth of rats receiving the sucrose diet (Diet 616) was very much superior to that of animals receiving the glyceryl laurate diet. In fact, the growth of animals receiving the glyceryl laurate is retarded from the beginning, whereas the animals receiving sucrose show normal growth at this time. About 30 per cent of the animals on the glyceryl laurate diet die very suddenly before the 50th day of life. The cause for the sudden death has not been ascertained.

Although ingested glyceryl laurate was well absorbed, nevertheless the product melted at 43°, which is slightly above the body temperature of the rat. 10 per cent ethyl laurate was accordingly incorporated with the glyceryl laurate, and the resulting mixture melted within the range of the rat's body temperature. There was, however, only slight improvement in its biological value.

Glyceryl laurate was then recrystallized from ethyl alcohol in the hope of removing any toxic substance, if such existed. Again, the improvement was only slight. The possibility that glyceryl laurate was toxic seemed unlikely, because the mere addition of small amounts of certain fatty materials (such as the ether extract of corn-starch fat) brought about a marked improvement which it is doubtful could have occurred in the presence of a toxic sub-

stance. We then endeavored to determine whether glycerides of other saturated fatty acids behaved in like manner. The glycerides of caprylic and myristic acids (also obtained from coconut oil) were fed in the same proportions and similar responses were obtained. Three synthetic coconut oils were then prepared and will be referred to as (1) synthetic coconut oil—prepared by saponification with 20 per cent potassium hydroxide, acidulation with sulfuric acid, collection and washing of the fatty acids with hot water, distilling in high vacuum, and reesterification with redistilled glycerol;<sup>1</sup> (2) “desoleosynthetic” coconut oil consisted of the glycerides of the individual fatty acids of coconut oil combined in the proportion in which they exist in coconut oil with the exception of the liquid fraction, which was omitted; (3) “oleosynthetic” coconut oil which consisted of the glycerides of the individual fatty acids of coconut oil combined in the proportion in which they exist in coconut oil. The composition of the oleosynthetic coconut oil and desoleosynthetic coconut oil is as follows:

Glyceride	Oleosynthetic coconut oil	Desoleosynthetic coconut oil
	<i>parts</i>	<i>parts</i>
Caprylin.....	8	8
Caprin.....	5	5
Laurin.....	51	51
Myristin.....	19	19
Palmitin.....	10	10
Liquid fraction of coconut oil.....	7	None

The animals receiving the oleosynthetic coconut oil were superior to those receiving the desoleosynthetic coconut oil and were about the same as those receiving the synthetic coconut oil. In short, there seemed outstanding the phenomenon that rats receiving the glycerides of saturated fatty acids as the sole source of energy were distinctly inferior to those receiving sucrose, unless unsaturated fatty acids were present in the diet.

The glycerides of saturated fatty acids used were separated from coconut oil in our fractionating columns (5) and esterified with

<sup>1</sup> This procedure is described in greater detail in a previous publication (1).



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glycerol at 200–230°, under constant agitation with a stream of CO<sub>2</sub>.

As sources of unsaturated fatty acids we used: (a) Starch fat obtained as a by-product in the manufacture of syrup from corn-starch.<sup>2</sup> The material as we received it was extracted with ether, the ether was then evaporated and the residue taken up in pentane. 1 cc. of the pentane solution carried 0.33 gm. of starch fat (labeled Supplement 36-c). (b) Liquid acids from corn oil after

TABLE I  
*Composition (in Gm.) of Diets*

Diet No.....	616	639	643	644	645	646	647	648	650	651	652	653
Casein (Van Slyke).....	24	40	40	40	40	40	40	40	40	40	40	40
Salt Mixture 185 (4).....	3.9	5	5	5	5	5	5	5	5	5	5	5
Sugar.....	72.1											
Glyceryl laurate.....		60										
Coconut oil.....			60									
Synthetic coconut oil.....				60								
Oleosynthetic coconut oil....					60							
Desoleosynthetic coconut oil						60						
Glyceryl laurate recrystal-							60					
lized.....								60				
Glyceryl laurate + 10 per									60			
cent ethyl laurate, m.p.										60		
39–40°.....											60	
Butter fat.....												60
Lard.....												
Glyceryl myristate.....											60	
“ caprylate.....												60

removal of as much of the oleic acid as possible by Farnsteiner's (6) method. This product consisted of about 90 per cent linoleic acid as calculated from its iodine number of 170, and will be referred to in this paper as linoleic acid. (c) "Pure" oleic acid prepared from commercial oleic acid by the method of Skellon (7).

### *Results of Feeding*

Female rats 21 days old were fed the various diets and maintained three in a cage on false screen bottoms. The components

<sup>2</sup> The starch fat is the centrifugal residue obtained from the Corn Products Company of Argo, Illinois, to whom we wish to express our thanks.

of the diets used are listed in Table I. All the diets were supplemented with the known necessary factors as follows: Vitamins A and D (8) were supplied as the non-saponifiable matter of cod liver oil, equivalent to 84 mg. of the oil daily. Vitamin E<sup>3</sup> was supplied as the non-saponifiable matter of wheat germ oil, equivalent to 500 mg. of the oil weekly. Vitamins B and G were supplied

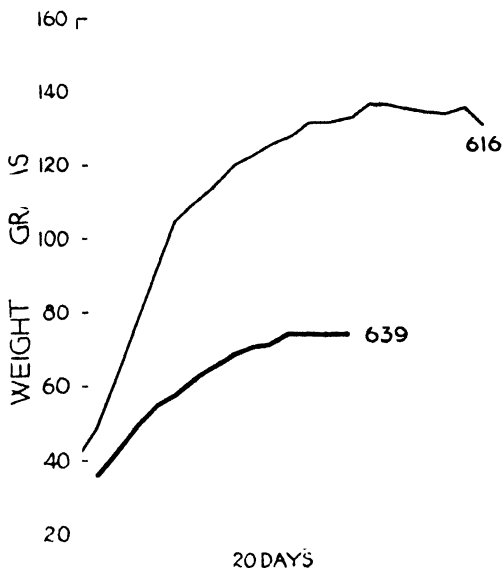


FIG. 1. Composite growth curves. The curve for Diet 639 (glyceryl laurate) represents the average growth of thirty-five animals. The curve for Diet 616 (sucrose) represents the average growth of fifteen animals.

by feeding 1 gm. daily of ether- (anhydrous) extracted yeast (Northwestern).

Fig. 1 shows the difference in growth obtained when diets containing glyceryl laurate (Diet 639) or sucrose (Diet 616) as their sole source of energy are compared.

Fig. 2 shows the relative responses to a series of natural fats, synthetic fats, and glycerides of saturated fatty acids. The

<sup>3</sup> Cf. the preceding article, Paper I, for the method of preparation of this material.

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natural fats fed were coconut oil (Diet 643), lard (Diet 651), and butter (Diet 650). The synthetic fats fed were synthetic coconut oil (Diet 644), oleosynthetic coconut oil (Diet 645), and desoleosyn-

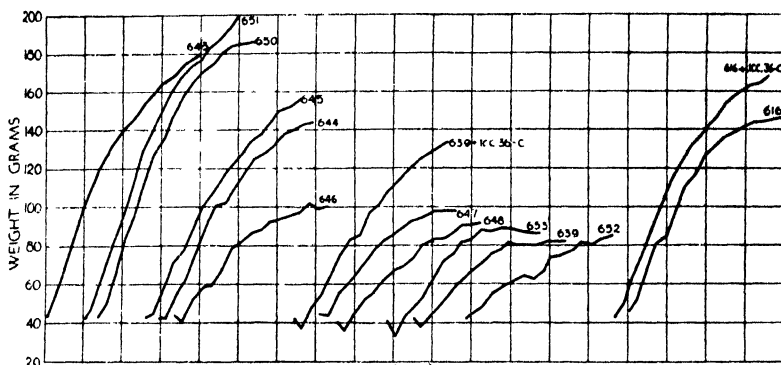


FIG. 2. Composite growth curves, each curve representing the average growth of six animals. See Table I for complete dietary ingredients. Supplement 36-c represents the fatty acid fraction from starch fat.

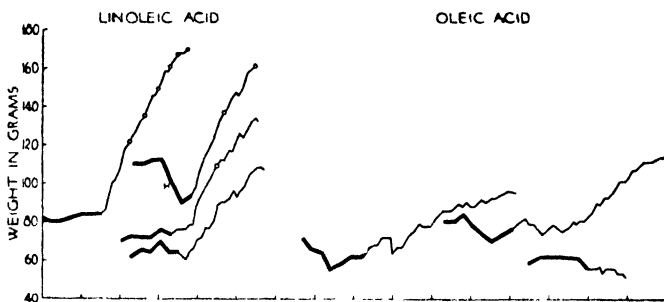


FIG. 3. Individual growth curves which show the effects of adding two different fatty acids to the glyceryl laurate diet. The heavy line indicates the 30 day period previous to the administration of the fatty acid. Small circles interrupting the curves indicate the occurrence of estrum. H indicates hematuria.

thetic coconut oil (Diet 646). The glycerides of saturated fatty acids were laurin (Diet 639), myristin (Diet 652), and caprylin (Diet 653). The natural fats are superior in every case to the synthetic fats. There is a sharp division in the synthetic fats

determined by the presence or absence of unsaturated fatty acids resulting in uniformly better growth in the rats. Contrast the growth obtained with oleosynthetic coconut oil (Diet 645) and that obtained with desoleosynthetic coconut oil (Diet 646). When unsaturated fatty acids (Diet 639 + 0.1 cc. of Supplement 36-c) are fed to animals receiving the glyceryl laurate diet (Diet 639), better growth is obtained than when the unsupplemented diet is fed. But even so, this improved growth does not for a long time equal that obtained with the unsupplemented sucrose diet (Diet 616),

Fig. 3 shows that unsaturated fatty acid fractions containing linoleic acid are very effective in improving the dietary mixture containing the glyceride of lauric acid as the sole source of energy, whereas a pure oleic acid preparation carefully freed as far as possible of linoleic acid is only slightly effective.

#### DISCUSSION

Burr and Burr have emphasized the importance of unsaturated fatty acids for rats when fed on fat-free diets where the sole source of energy was sucrose. On such diets rats grow normally for some time and ultimately attain a weight of 135 to 145 gm. When diets carry glycerides of saturated fatty acids as the sole source of energy, approximately a third of the animals succumb before the 50th day of life. In the surviving group an average attainment of 75 to 90 gm. of body weight results, *normal growth being at no time obtained*. This early and profound stunting of growth suggests that we have to do with some factor or factors different from those involved when sucrose is the sole source of energy. There is other evidence that the physiological condition of the animal on these two types of diet is not the same. The actual water consumption *per diem* per animal is less on the high fat than on the sucrose-casein diet. With the sugar diet, Burr and Burr have shown that the curative substances (unsaturated fatty acids) reduce the abnormally high water intake *per diem* per animal, although with the resumption of growth increased water consumption might be expected. When the curative materials are fed to animals on the fat diets, the resulting resumption of growth is not accompanied by appreciable further reduction in water intake.

## SUMMARY

1. Animals do not thrive on diets containing the essentials hitherto known (adequate amounts of protein, all of the known vitamins, and the essential inorganic constituents), but in which the energy requirements are met by the glycerides of saturated fatty acids.

2. Glycerides of saturated fatty acids when fed as the sole source of energy, do not promote at any time growth in the rat equal to that obtained with sucrose as the sole source of energy.

3. Unsaturated fatty acid preparations containing fatty acids with more than one double bond markedly improve such diets.

4. Oleic acid, freed of double bond fatty acids, produces a very slight response, whereas linoleic acid produces a marked response.

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## THE SPARING ACTION OF FAT ON VITAMIN B

### II. THE RÔLE PLAYED BY THE MELTING POINT AND THE DEGREE OF UNSATURATION OF VARIOUS FATS\*

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#### INTRODUCTION

In a previous communication (1) we reported that fats, in the absence of vitamin B, were able in part to compensate for the deficiency of this vitamin. At that time we reported the effectiveness of lard and cottonseed oil. We thought it important to determine whether the sparing action of a fat was in any way related to its physical properties, such as melting point and degree of unsaturation.

#### *Fats Used and Their Preparation*

The fats used in this investigation are listed in Table I. The cottonseed oil was bought on the market under the trade name of Wesson oil and the partially hydrogenated oil under the name of Crisco. We prepared the fully hydrogenated cottonseed and synthetic cottonseed oils from Wesson oil.

Perilla<sup>1</sup> oil was incorporated in our series because of its unusually high degree of unsaturation. It is obtained from the seeds of an annual plant, *Perilla ocimoides*, and is more unsaturated than linseed oil. We partially hydrogenated one lot of this oil and one lot we hydrogenated fully. With the oils mentioned above and

\* Aided by grants from the Committee for Research in Problems of Sex of the National Research Council and from the Rockefeller Foundation. These funds have been generously augmented by the Board of Research and the College of Agriculture of the University of California.

<sup>1</sup> We are grateful to S. L. Jones and Company of San Francisco for the oil used in these experiments.

their derivatives we were able to study the effect of fats with various degrees of unsaturation. We could also study the effect of low, intermediate, and high melting points.

While the cottonseed and perilla oil preparations enabled us to study fats of a high degree of unsaturation with low melting points or of low unsaturation with high melting points, we were desirous of securing a fat with a low melting point which at the same time was of low unsaturation. Coconut oil served this purpose admirably because even after complete hydrogenation it still possessed a melting point below the body temperature of the rat. The synthetic coconut oil was prepared in the same manner as the synthetic cottonseed oil. These synthetic oils gave us products

TABLE I  
*Melting Points and Iodine Numbers of Fats Used*

	Melting point	Iodine No.
Cottonseed oil. ....	Liquid	107.5
Partially hydrogenated cottonseed oil . . . . .	38.0	80.0
Hydrogenated cottonseed oil . . . . .	62.0	1.0-1.5
Synthetic " " . . . . .	Liquid	105.0-110.0
Perilla oil . . . . .	"	187.0
" " partially hydrogenated. . . . .	42.0	72.0
" " almost completely hydrogenated. . . . .	67.5	1.0-2.0
Coconut oil. . . . .	25.0	7.0-8.0
Hydrogenated coconut oil. . . . .	35.0	0-0.2
Synthetic " " . . . . .	27.0	5.0
Lard. . . . .	34.0	60.0

free from vitamin B, as well as the greater part of the unsaponifiable matter.

The fats were hydrogenated<sup>2</sup> in a small metal converter of about a liter capacity. The converter had a metal stirrer which entered through a packing gland, making it possible to hydrogenate under pressure. The hydrogenation was carried on at 15 pounds pressure and at a temperature of 170°. We used a nickel catalyst mixed with filter cell in such an amount that about 0.2 per cent of metallic nickel<sup>3</sup> was used. The course of the hydrogenation

<sup>2</sup> We wish here to thank Mr. Dean C. Ingraham and Durkee Famous Foods for the use of the converter employed in the process of hydrogenation.

<sup>3</sup> The catalyst was prepared by reducing a mixture of purified NiCO<sub>3</sub>.

was followed by making melting point determinations and generally was complete in 2 to 3 hours after the temperature was reached at which hydrogenation occurs. At times, when for unknown reasons the catalyst was not very active, 6 to 8 hours were required for complete saturation. Increasing the amount of catalyst always speeded up a sluggish hydrogenation.

The synthetic cottonseed and coconut oils were prepared by saponification with 20 per cent KOH, acidulation with  $H_2SO_4$ , collection and washing of the fatty acids with hot water, distillation in a high vacuum, and reesterification with redistilled glycerol. This procedure is described in greater detail in a previous publication (1). The iodine number of the synthetic cottonseed oil was about the same as that of the untreated cottonseed oil. The iodine number of the synthetic coconut oil was slightly lower than that of the untreated coconut oil. This is due to the small residue containing unsaturated fatty acids, which is always left after the distillation of the coconut oil fatty acids. The amount of unsaturated fatty acids in coconut oil is very much less than in cottonseed oil, and the loss of a small amount in the residue of the coconut oil is, therefore, reflected by its lower iodine number.

#### *Determination of Fat Absorbed*

To judge properly the sparing action of any fat it was necessary to make certain that it was fully absorbed. This was accomplished by keeping records of the food consumed, and collecting, drying (at  $90^\circ$ ), and determining the fat content of the feces. The intake of fat was calculated on the basis of the fat mixed in the diet.

The problem of determining the fat in the feces deserves some attention here. Much work has been done on this subject (2-4) and there are many recommendations as regards methods and solvents used, but a specific, yet simple and accurate method has not been worked out. One of the methods often recommended consists in the saponification of the fecal fatty materials, isolation of the fatty acids, and the determination of the amount present

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and filter cell with hydrogen at a temperature of about  $315^\circ$ . The proportions of  $NiCO_3$  and filter cell used were such as to give about 1 part of metallic nickel to 1 part of filter cell in the catalyst.



by either titration or weighing. Such a procedure is recommended when there are few determinations to be made. Another method which involves the direct extraction of the fat from the feces entails certain inaccuracies; namely, the insolubility of certain soaps in the solvent, and the extraction of materials not fat. Since we merely wished to determine the approximate degree of absorption obtained, we concluded that extracting the feces with hot benzene and weighing the extract would give us the desired accuracy with the least expenditure of labor. Benzene was chosen because it is not only non-polar, but is easy to obtain water-free and is not difficult to handle. The extraction was carried out in alundum thimbles in an apparatus of the type recommended by the Joint Rubber Insulation Committee (5). In a preliminary experiment, the determination of fat in the feces by the method of saponification according to Sperry and Bloor (4) was compared with benzene extraction. For our purpose the agreement was satisfactory; namely, 11.7 per cent by the method of benzene extraction and 13.3 per cent by the saponification method. The lower figure given by benzene extraction need not be the more inaccurate since the petroleum ether extract obtained by the saponification method most certainly contained substances not fatty acids.

In diets containing large amounts of unabsorbable fats, there existed the possibility that large amounts of insoluble soaps in the feces would escape extraction by pure dry benzene. Analysis of such extracted feces failed to disclose a higher per cent of residual soaps than was found with normal diets.

#### *Feeding Experiments*

The diets used are listed in Table II. The oils that were liquid at room temperature were simply mixed into the diet. The fats that were solid at room temperature were melted and mixed with the diet. When the fully saturated cottonseed and perilla oils were so treated, the fats solidified in hard flakes, and to avoid this we recrystallized the two fats from ether and thus obtained a very finely divided product which could be mixed quite easily with the other ingredients of the diet. All the diets were supplemented with 2 drops of cod liver oil daily so that the "fat-free" diet carried not only this fat but also that in the casein and yeast. The fats

from these sources are not included in the figures indicating per cent of fat in the diet. Food consumption records were kept, and collections of feces were made daily for five periods of 2 weeks each for the cottonseed oil series and for two periods of 2 weeks each for the other series. The differences in the data of the various

TABLE II  
*Composition (in Gm.) of Diets*

	Diet No.																	
	543	563	564	565	566	567	568	569	570	571	572	573	574	580	583	584	585	586
Casein (L-3)*	20	23	23	23	23	30	30	30	30	38	38	38	38	30	30	30	30	30
Sugar	70	56	56	56	56	41	41	41	41					41	41	41	41	41
Salt Mixture 185†	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Autoclaved yeast‡	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
Partially hydrogenated cottonseed oil		10				25				55								
Cottonseed oil			10				25				55							
Synthetic cottonseed oil				10				25				55						
Hydrogenated cottonseed oil					10				25				55					
Lard														25				
Coconut oil															25			
Synthetic coconut oil																25		
Hydrogenated coconut oil																	25	
Perilla oil																		25
“ “ partially hydrogenated																		25
Perilla oil almost completely hydrogenated																		25

\* See (1).

† McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, **33**, 63 (1918).

‡ The whole dried yeast was generously supplied us by the Fleischmann Laboratories of Standard Brands Incorporated.

periods were not marked, so only the totals are given for the sake of brevity. The time during which these records were kept is marked off on the composite growth curves shown in Figs. 1 to 3. Composite growth curves are presented since the three animals of each group were maintained in one cage and were treated as a

unit both as regards the amount of food consumed and the feces collected. Litter mate sisters were given the various diets when 21 days old and were kept on wire screen bottoms for the duration of the experiment. The cottonseed oil fats were fed at levels of 9.7, 22.7, and 51.4 per cent of the diet. Each level of fat was fed with additions of 50, 200, and 800 mg. of a brewers' yeast of tested vitamin content.<sup>4</sup> Sixteen groups of rats were therefore used.

The perilla and coconut oil series were not so extensive as the cottonseed oil series. We employed here one level of fat (22.7 per cent) and only two yeast levels (50 mg. and 1 gm.). The food consumption records and feces collection records were kept for two periods of 2 weeks each, as designated on the composite growth curves.

#### *Cottonseed Oil Series*

*50 Mg. of Yeast*—The composite growth curves for the cottonseed oil series are given in Fig. 1. At the low level of yeast (50 mg.) there is definite evidence of sparing by all fats of the cottonseed oil series except the fully hydrogenated cottonseed oil. At the level of 9.7 per cent fat superior growth resulted from the use of synthetic cottonseed oil. Fully hydrogenated cottonseed oil gave no evidence of sparing action. From an examination of Table III, which shows the utilization of the various fats, we see that while the other fats are well utilized the fully hydrogenated cottonseed oil (Diet 566) is not so well utilized. About half the solid fat was recovered in the feces, and this no doubt accounts for the poor growth obtained on the diet containing this fat.

At the 22.7 per cent level of fat there was no particular superiority of one fat over another since all showed decided sparing action, with the exception of the diet containing fully hydrogenated cottonseed oil which induced very poor growth and the early death of the rats. On this high level of fully hydrogenated cottonseed oil the feces were often white, containing about 75 per cent of fat, and the amounts eliminated were considerably greater than on the lower level of the fat.

<sup>4</sup> We desire to thank President R. M. Allen and Dr. Edward A. Rumley of the Vitamin Food Company of New York for generously supplying a whole dried brewers' yeast remarkably high in its content of the antineuritic vitamin B.

At the 51.4 per cent level of fat, the rats on fully hydrogenated cottonseed oil died very quickly, and so play no part in this discussion. The other fats all showed a decided sparing action, the partially hydrogenated cottonseed oil being the best, cottonseed oil next, and synthetic cottonseed oil the worst. The cottonseed oil caused a slight diarrhea at this high level of feeding, which cleared up in the course of the experiment. The synthetic cotton-

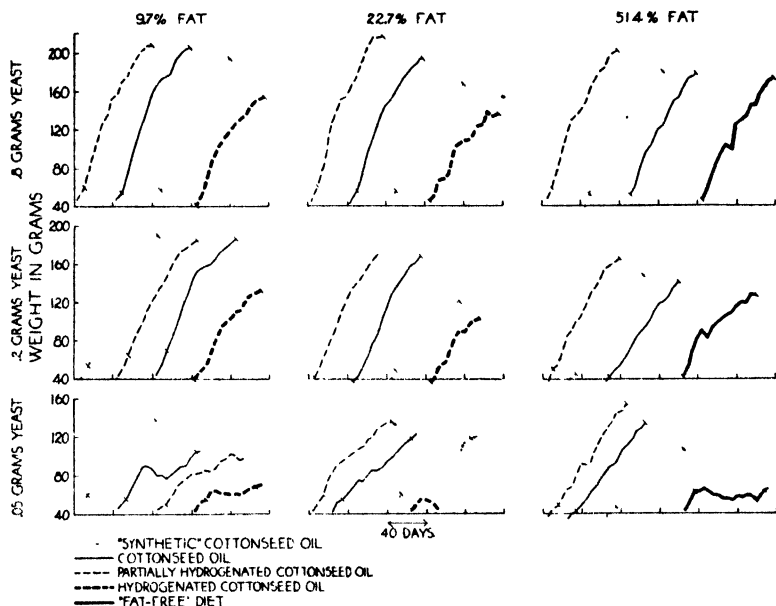


FIG. 1. Each curve represents the average growth of three animals. The small lines crossing the curve indicate the period during which determinations were made of the amount of fat consumed and the amount of fat absorbed. The diets were supplemented with 2 drops of cod liver oil daily.

seed oil resulted in a severe diarrhea which lasted throughout the period under investigation. In spite of the diarrhea the animals grew remarkably well. The quantity of the feces excreted by these rats was surprisingly small and was associated with a remarkably low fat content, indicating that almost all the fat taken in was absorbed. If it were possible to correct for the fatty material excreted in the intestine, we could probably conclude that all the fat had been absorbed.

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TABLE III  
*Utilization of Fat in Cottonseed Oil Series*

Diet No.	Increase in body weight	Food intake	Fat intake	Feces excreted	Fat in feces	Fat absorbed	
	gm.	gm.	gm.	gm.	gm.	gm.	per cent
50 mg. yeast							
563	162	1048	101.9	34.5	8.4	93.8	92.0
564	136	1134	110.1	37.8	5.3	104.6	95.0
565	238	1420	137.7	44.3	6.1	131.6	95.0
566	56	911	88.4	74.2	44.6	43.8	49.0
567	232	1310	297.7	74.5	12.6	285.0	96.0
568	199	1303	296.1	41.6	9.9	286.2	97.0
569	186	1287	292.7	33.1	2.9	289.8	99.0
570*		452	43.8	58.3	42.8	1.0	2.0
571	249	1016	522.1	43.8	13.7	511.4	98.0
572	267	1027	527.9	59.6	19.1	508.8	97.0
573	184	757	389.1	26.3	7.5	381.6	98.0
542		1322		32.3	4.2		
200 mg. yeast							
563	358	1846	181.6	77.1	19.4	164.9	91.0
564	376	1950	189.1	72.6	9.2	179.9	95.0
565	406	1828	177.3	92.9	8.2	169.1	95.0
566	266	1622	157.2	145.3	98.1	59.1	37.5
567	369	1694	385.0	67.0	22.9	362.1	94.0
568	387	1719	390.6	62.7	12.9	377.7	97.0
569	218	1343	305.2	34.5	4.3	300.9	98.0
570	145	1413	321.2	297.2	231.4	89.8	28.0
571	398	1147	589.5	49.7	14.1	575.3	98.0
572	298	979	505.5	37.6	11.1	492.4	97.0
573	291	849	436.0	21.7	3.4	432.6	99.0
542		1722		58.9	5.5		
800 mg. yeast							
563	453	2208	216.8	90.1	18.0	196.2	90.0
564	440	2034	197.4	98.0	11.7	185.7	94.0
565	394	2004	194.4	91.4	10.0	184.4	95.0
566	330	1961	190.1	225.2	146.4	49.6	26.0

TABLE III—*Concluded*

Diet No.	Incre- ment in body weight	Food intake	Fat intake	Feces excreted	Fat in feces	Fat absorbed	
	gm.	gm.	gm.	gm.	gm.	gm.	per cent
567†	282	1501	340.7	79.3	24.8	316.0	93.0
568	443	1703	387.3	70.6	12.6	374.0	96.0
569	329	1508	343.0	39.7	5.6	337.4	98.0
570	252	1843	419.0	315.1	248.5	170.5	40.0
571	415	1242	634.9	59.4	18.4	620.0	98.0
572	401	1144	588.0	52.9	15.4	572.6	97.0
573	360	1006	517.3	30.1	5.2	512.8	99.0
542		2376		95.3	10.1		

The figures represent the conditions prevailing during the periods which are marked off on the growth curves. The original data consisted of five consecutive periods of 2 weeks each, but since there was very little variation in the groups, we present only the totals here.

\* These data represent only the first three periods because the animals died during the fourth period.

† Figures represent the last four periods.

*200 Mg. of Yeast*—Definite sparing action of fat for vitamin B was demonstrated when 200 mg. of yeast were fed with a diet containing 9.7 per cent fat, indicating that 200 gm. of yeast did not satisfy the rat's requirements for vitamin B. When the fat content of the diet was increased to 22.7 per cent, there was no improvement in growth over that obtained on the 9.7 per cent level as was the case when only 50 mg. of brewers' yeast were fed. At the 200 mg. level of yeast 9.7 per cent of fat was sufficient to give the maximum sparing action possible by fat addition. As was expected, very poor growth was obtained with the fully hydrogenated cottonseed oil, but to our surprise, the synthetic cottonseed oil, which gave such superior results at the level of 9.7 per cent, here gave very poor results. These inferior results cannot be accounted for by poor absorption because it is even slightly better than is the case with the other fats.

At the highest level of fat, 51.4 per cent, the partially hydrogenated cottonseed oil gave the best results. Here again we noticed a slight diarrhea with cottonseed oil and a severe diarrhea with synthetic cottonseed oil. The absorption of the fat was just

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as good when fed as 51.4 per cent of the diet as when fed as 9.7 per cent; in fact, the per cent absorbed was actually higher when the larger amounts were fed.

*800 Mg. of Yeast*—When an adequate amount of yeast was fed, the fat diets yielded superior results at the level of 9.7 per cent with the exception of the diet carrying fully hydrogenated cottonseed oil. The animals on this diet were actually in poorer condition than animals with no added fat in their diet. 74 per cent of the fat fed was recovered in the feces.

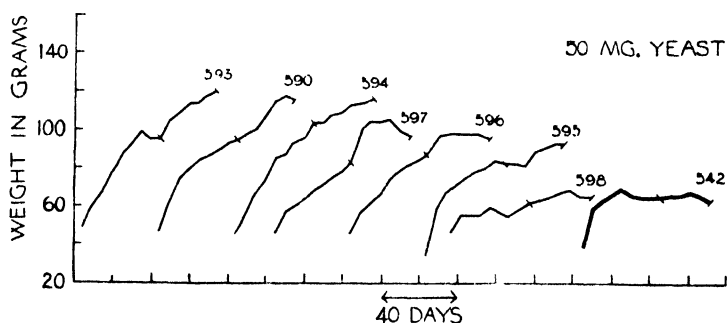


FIG. 2. Each curve represents the average growth of three animals. The small lines crossing the curve indicate the period during which determinations were made of the amount of fat consumed and the amount of fat absorbed. The diets contained 25 per cent of fat in the following order: Diet 590 lard, Diet 593 coconut oil, Diet 594 synthetic coconut oil, Diet 595 hydrogenated coconut oil, Diet 596 perilla oil, Diet 597 partially hydrogenated perilla oil, Diet 598 perilla oil almost completely hydrogenated. Diet 542 did not contain added fat. All the diets were supplemented with 50 mg. of yeast and 2 drops of cod liver oil daily.

At the level of 22.7 per cent fat we again found synthetic cottonseed oil produced quite poor growth. We are at a loss to understand it.

When the diet contained 51.4 per cent fat, the partially hydrogenated cottonseed oil was superior to synthetic cottonseed oil; the animals receiving the untreated cottonseed oil grew slightly better than the animals on the fat-free diet. The rats developed a slight diarrhea on cottonseed oil and a severe diarrhea on synthetic cottonseed oil. Considering the severity of the diarrhea, the rats grew remarkably well.

*Perilla and Coconut Oils*

Since the diets containing perilla and coconut oils were fed at the same time, with litter mate sisters distributed between the two groups, we shall present the data together.

*50 Mg. of Yeast*—All the fats were fed at the level of 22.7 per cent, and show a definite sparing action with the exception of the fully hydrogenated perilla oil (Diet 598), (Fig. 2). This can be

TABLE IV  
*Utilization of Fat in Perilla and Coconut Oil Series*

Diet No.	Incre- ment in body weight	Food intake	Fat intake	Feces excreted	Fat in feces	Fat absorbed	
	gm.	gm.	gm.	gm.	gm.	gm.	per cent
50 mg. yeast							
590	68	363	82.4	27.8	5.7	76.7	93.0
593	37	446	101.4	15.8	1.9	99.5	98.0
594	40	584	132.6	22.6	2.6	130.0	98.0
595	30	433	98.7	18.9	4.0	94.7	96.0
596	26	478	108.6	20.4	3.5	105.1	97.0
597	45	545	123.8	27.5	10.1	113.7	92.0
598	16	423	96.1	103.7	85.5	11.0	11.0
542	-3	559		16.8	0.9		
1 gm. yeast							
590	139	1073	243.9	49.6	10.9	232.9	96.0
593	123	912	207.5	50.0	10.4	197.1	95.0
594	154	827	188.0	44.6	5.3	182.7	97.0
595	104	887	201.4	54.4	14.3	187.1	93.0
596	122	796	180.9	43.3	5.7	175.2	97.0
597	105	938	213.1	57.4	21.9	191.2	90.0
598	69	996	226.3	264.8	223.6	2.7	1.0
542	89	957		57.7	8.0		

explained by the very poor absorption of this oil (Table IV). The other fats show good absorption with the exception of the lard (Diet 590) and partially hydrogenated perilla oil (Diet 597) which were absorbed somewhat less completely than the others.

*1 Gm. of Yeast*—At this high level of yeast the growth of the animals on the diet containing the fully hydrogenated perilla oil (Diet 598) was inferior to that of those on the fat-free diets (Fig. 3). The diets containing perilla oil (Diet 596), the partially hydro-



genated perilla oil (Diet 597), and hydrogenated coconut oil (Diet 595) produce about the same growth as the fat-free diet (Diet 542). The coconut oil (Diet 593), synthetic coconut oil (Diet 594), and lard (Diet 590) were superior to the fat-free Diet 542. The fully hydrogenated coconut oil and partially hydrogenated perilla oil were not so completely absorbed as were the other fats.

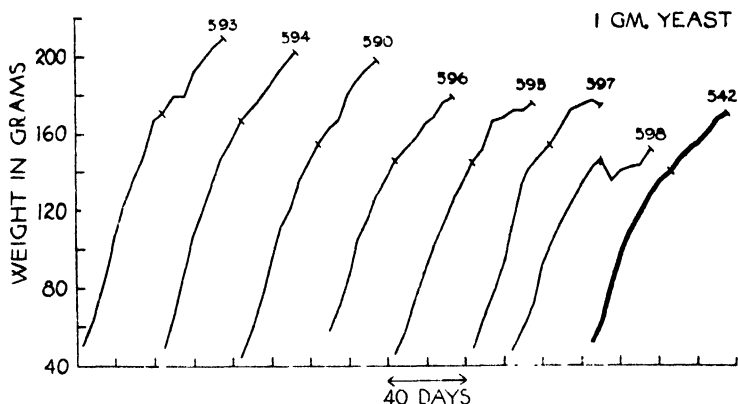


FIG. 3. Each curve represents the average growth of three animals. The small lines crossing the curve indicate the period during which determinations were made of the amount of fat consumed and the amount of fat absorbed. The diets contained 25 per cent of fat, as given in the legend to Fig. 2. All the diets were supplemented with 1 gm. of yeast and 2 drops of cod liver oil daily.

#### DISCUSSION

It is clear that all the fats studied, with the exception of those whose melting points are so high that they are not fully absorbed, demonstrate the phenomenon of sparing vitamin B. There is no striking difference in their ability to do this, which is dependent on either the melting point or the iodine number.

The absorption and utilization of fats as determined by the growth produced in rats is apparently not interfered with by drastic treatment such as hydrogenation under high temperature (170°), pressure (15 pounds), and subjection to saponification with strong alkali, distillation, and esterification with glycerol to temperatures to 230°. Hydrogenation seems to lower somewhat the coefficient of absorption of fats, but not enough to affect materially

their biological value, provided the melting point is kept relatively near that of the rat's body temperature. The subsection of fats to the manipulations involved in the production of synthetic fats seems actually to improve their biological value when fed at low levels (9.7 per cent). When fed at high levels (51.4 per cent), an unaccountable diarrhea develops, without, however, any appreciable increase in the amount of fat passing into the feces. Almost in every case, the coefficient of absorption of the synthetic fat is greater than the natural fat. As a suggestion, this may be due to the fact that the synthetic fat consists only of the actual glycerides.

#### SUMMARY

1. It has been previously shown that the liberal inclusion of fat in the diet will enable an animal to withstand for many weeks the withdrawal or omission of the antineuritic vitamin B, and in the presence of fat more growth will occur at any definite level of vitamin B than is the case with a fat-free diet.

2. Provided they melt near body temperature, neither the precise melting point nor the degree of saturation of fats plays an important rôle in their remarkable ability to spare the amount of vitamin B required for any definite growth performance.

3. Fats melting at 62° or above are very poorly absorbed by the rat and are unable to exhibit the above sparing action. Fats melting at 38° spare just as successfully as those which are liquid at room temperature.

4. Fats almost saturated and possessing an iodine number of 8 spare as well as those highly unsaturated, with an iodine number of 187.

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## THE SPARING ACTION OF FAT ON VITAMIN B

### III. THE RÔLE PLAYED BY GLYCERIDES OF SINGLE FATTY ACIDS\*

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#### INTRODUCTION

The natural fats are known to be made up of many different kinds of fatty acids combined with glycerol in various ways. In addition, they carry in solution various known and unknown materials, such as are found in the unsaponifiable matter. Up to this time the most simplified form of fat used in our studies on the sparing action of fat on vitamin B (1) consisted of the total fatty acids of a given fat distilled and reesterified with redistilled glycerol. This so called synthetic fat nevertheless remained a complex mixture of various fatty acids, though it was by no means so complex as the natural fat from which it was derived. We felt that a deeper insight could be had into this problem by the use of fats consisting of the glycerides of single fatty acids. Our attempts to carry out this project form the subject of this communication.

#### *Preparation of Glycerides of Single Fatty Acids*

Fatty acids were isolated in pure form by fractional crystallization and distillation. Coconut oil was the source of the pure caprylic, capric, lauric, and myristic acids. Palmitic acid was obtained from bayberry wax, while stearic acid was obtained on the market and further purified in our laboratory by a single distillation in a Claisen flask.

\* Aided by grants from the Committee for Research in Problems of Sex of the National Research Council and from the Rockefeller Foundation. These funds have been generously augmented by the Board of Research and the College of Agriculture of the University of California.

Caprylic, capric, lauric, and myristic acids were separated in the fractionating column previously described (2). We started with the fatty acids of coconut oil, isolated by saponification with alcoholic KOH, distillation of alcohol, acidification, washing of the fatty acids, and subjecting them to a preliminary distillation from a Claisen flask. Special care was taken to remove all the alcohol from the soaps before preliminary distillation, otherwise some ethyl esters would form and thus render a separation of the individual fatty acids in the column impossible. This was accomplished by addition of water to the soaps and evaporation on the steam bath. The details of this work will be published separately.

TABLE I  
*Acetyl Values and Proportions of Di- and Triglycerides*

Glyceride	Acetyl value	Calculated per cent of diglycerides	Calculated per cent of triglycerides	Saponification No.	Calculated saponification No.
Stearin.....	57.8	68.7	31.3	180.0	182.0
Palmitin.....	65.9	71.0	29.0	200.0	200.3
Myristin.....	44.6	43.8	56.2	226.5	227.0
Laurin.....	85.0	21.1	74.9	250.0	257.0
Caprin.....	133.0	34.9	65.1	291.0	294.0
Caprylin.....	35.7	8.2	91.8	325.0	356.0
Glycerol.....	772.0				

The esterification was carried on in an oil bath at about 200° with approximately a 50 per cent excess of glycerol. Carbon dioxide was vigorously bubbled through the mixture for purposes of agitation and the removal of the water vapor formed. The esterification was completed by heating in a high vacuum at 220-230°. By this method, 95 to 98 per cent of the free fatty acids was esterified.

Palmitic acid was prepared from bayberry wax according to the method of Chittenden and Smith (3). It was recrystallized from alcohol and finally fractionated in our column. The commercial stearic acid was redistilled in our Claisen flask and used without further purification.

The glycerides were acetylated and the acetyl numbers deter-

mined, and from these we calculated the character of the glycerides present. We found them to be a mixture of di- and triglycerides. Table I gives the acetyl values and the calculated proportions of di- and triglycerides.

The saponification numbers of the di- and triglycerides as calculated from the acetyl values agree very well with saponification numbers of the glycerides obtained upon analysis. There is one exception, however; namely, the considerable difference between the calculated saponification number and that actually determined in the case of caprylin. This may be due to anhydride formation, which, though in all probability small, could exert such an effect.

The melting points of the glycerides as determined in our laboratory are given below.

	°C.		°C.
Caprylin.....	7-8	Palmitin.....	58-60
Caprin.....	25-26	Stearin ...	58-59*
Laurin.....	43	Ethyl stearate .....	25-26*
Myristin .....	53-54	“ palmitate.....	19-20

\* The stearic acid was not pure; it contained both palmitic and oleic acids.

The degree of purity of the fatty acids was determined by their neutralization values.

### *Plan of Experiment*

The glycerides were fed in the diet at the level of 22.7 per cent, with no vitamin B added, and with two levels of vitamin B, namely, 50 mg. and 1 gm. of yeast.<sup>1</sup> The composition of the diets is given in Table II. The diets were supplemented with 2 drops of cod liver oil daily so that all the diets carried not only this fat but also that in the casein and the yeast. The fats from these sources are not included in the figures indicating the per cent of fat in the diet. In addition to the glycerides, the ethyl esters of palmitic and stearic acids were fed. This was important because

<sup>1</sup> A whole dried brewers' yeast was used. We are grateful to President R. M. Allen and to Dr. Edward A. Rumley of the Vitamin Food Company of New York for the supply of yeast.

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of the incompleteness of absorption of the glycerides of palmitic and stearic acids, particularly the glyceride of stearic acid. We related the incompleteness of absorption of these glycerides to their elevated melting points and thought it of importance to feed these fatty acids in a form liquid at body temperature. The ethyl esters adequately served this purpose. Cottonseed oil was the native fat used as the control.

The fat content of the feces was determined by benzene extractions according to the method described in Paper II of this series.

TABLE II  
*Composition (in Gm.) of Diets*

Diet No. . . . .	600	601	602	603	604	605	608	609	542	568
Casein (L-3)*. . . . .	30	30	30	30	30	30	30	30	20	30
Salt Mixture 185† . . . .	4	4	4	4	4	4	4	4	4	4
Sugar. . . . .	41	41	41	41	41	41	41	41	70	41
Autoclaved yeast‡. . . . .	10	10	10	10	10	10	10	10	10	10
Stearin. . . . .	25									
Palmitin. . . . .		25								
Myristin. . . . .			25							
Laurin. . . . .				25						
Caprin. . . . .					25					
Caprylin. . . . .						25				
Ethyl palmitate . . . . .							25			
“ stearate. . . . .								25		
Cottonseed oil. . . . .										25

\* See (1).

† McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, **33**, 63 (1918).

‡ The whole dried yeast was generously supplied us by the Fleischmann Laboratories of Standard Brands Incorporated.

The fat intake was calculated on the basis of the food consumed. In this manner, fat balances were obtained and the completeness of absorption of each glyceride was determined. These records were kept during the last 6 weeks of the experiment; that is, from the 58th to 100th day of the life of the animal. Each growth curve depicts the average growth of the four animals. Since the four animals of each group were maintained in the same cage, the data for food consumption and feces represent the total for the group; they are presented in Table III.

TABLE III  
*Utilization of Fat in Diet*

Diet No.	Incre- ment in body weight	Food intake	Fat intake	Feces excreted	Fat in feces		Fat absorbed	
	gm.	gm.	gm.	gm.	gm.	per cent	gm.	per cent
<b>No yeast</b>								
600*	†	180	40.9	32.0	23.0	72.0	17.9	43.8
601†	†	434	98.5	28.2	15.5	55.0	83.0	84.2
602	254	1596	362.3	62.1	23.1	37.1	339.2	93.5
603	46	977	221.8	35.1	5.8	16.5	216.0	97.5
604‡	33	804	182.5	31.6	4.0	12.6	178.5	98.0
605	249	1786	405.4	42.6	4.6	10.8	400.8	99.0
608†	†	797	180.9	56.8	24.1	42.4	156.8	87.0
609*	†	241	54.7	35.4	23.9	67.6	30.8	56.2
568†	†	354	80.3	11.8	2.5	21.1	77.8	97.0
542*	†	202		8.9	0.6		6.7	
<b>50 mg. yeast</b>								
600	65	1149	260.8	195.4	135.4	69.6	125.4	48.0
601	213	1578	358.2	101.5	58.4	57.4	299.8	83.5
602	242	1472	334.1	61.3	20.5	33.4	313.6	93.5
603	250	1336	303.2	39.5	7.4	18.7	295.8	97.5
604	234	1645	373.4	54.4	7.6	14.0	365.8	98.0
605	314	1850	419.9	64.2	7.1	11.0	412.8	98.5
608	173	1693	384.3	91.9	44.4	48.4	339.9	88.5
609†	†	464	105.3	73.9	50.0	67.6	55.3	52.4
568	358	1922	436.2	79.0	20.7	26.4	415.5	95.0
542	60	1493		37.4	3.2			
<b>1 gm. yeast</b>								
600	273	2148	487.6	387.9	292.4	75.6	195.2	40.0
601	294	1931	438.3	191.9	116.8	61.0	321.5	73.4
602	242	1756	398.6	113.6	37.1	32.6	361.5	91.0
603	280	1313	298.0	60.6	11.1	18.3	286.9	96.0
604	257	1729	392.5	81.1	13.7	16.8	378.8	96.5
605	340	2211	501.9	96.1	11.6	12.0	490.3	97.5
608	389	2049	465.1	189.0	109.5	58.0	355.6	76.4
609	120	1446	328.2	235.1	147.7	63.0	180.5	55.0
568	495	2119	481.0	95.9	21.1	21.8	459.9	95.5
542	363	3005		81.1	6.8			

The data were collected in three consecutive 2 week intervals, but for the sake of brevity, only the total is given and it represents the period of the last 6 weeks on the growth curve.

\* All the animals in this group were dead within the second period, and the figures therefore represent the performance during the first period.

† The growth is not given in this instance due to the fact that the weight of the animal was not always obtained on the day of death.

‡ All the animals in this group were dead within the third period, and the figures therefore represent the performance during the first and second periods.

§ Figures represent the performance of two animals.



*Results*

The growth curves obtained when animals received the individual glycerides are given in Figs. 1 to 3.

The results obtained in the absence of any added vitamin B are given in Fig. 1. Striking differences are seen at once. Superior performance was obtained with the diets containing either myristin (Diet 602) or caprylin (Diet 605). The animals averaged over 100 gm. in weight and were in fairly good condition at the end of the experiment, at which time the animals were 100 days

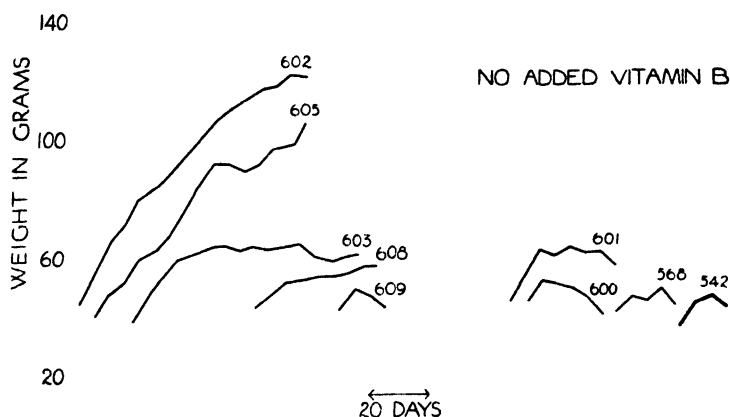


FIG. 1. Each curve represents the average growth obtained with four animals when no *vitamin B* was added to diets containing 25 per cent of the glycerides in the following order (see Table III for detail): Diet 600 stearin, Diet 601 palmitin, Diet 602 myristin, Diet 603 laurin, Diet 604 caprin, Diet 605 caprylin, Diet 608 ethyl palmitate, Diet 609 ethyl stearate, Diet 568 cottonseed oil, Diet 542 no fat. All the diets were supplemented with 2 drops of cod liver oil daily.

old. Such good growth had at no time been obtained with this amount of any natural fat, and, indeed, the cottonseed oil diet (Diet 568), used as the control, gave the usual poor performance obtained with natural fats in the absence of vitamin B.

Growth of the animals on the diets containing caprin (Diet 604) and laurin (Diet 603) was fairly good for this type of diet. Although the animals receiving laurin did not grow very much, they were alive at the end of the experiment at the age of about

100 days. Two of the animals on the diet containing caprin died, while two survived and were in fairly good condition at 100 days of age.

The palmitin diet (Diet 601) caused slightly better growth than the stearin diet (Diet 600) and was about as effective as the ethyl palmitate diet (Diet 608).

Summing up the results as determined by growth, we can say that there are definite biological differences among the glycerides of single fatty acids in the absence of vitamin B. Some of the

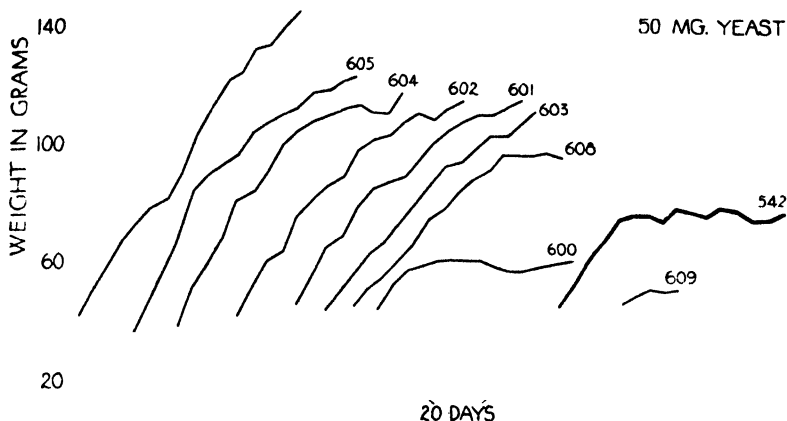


FIG. 2. Each curve represents the average growth obtained with four animals when 50 mg. of yeast were added to diets containing 25 per cent of the glycerides (see Table III for detail) in the order stated in the legend to Fig. 1. All the diets were supplemented with 2 drops of cod liver oil daily.

glycerides of single fatty acids are greatly superior to a natural fat like cottonseed oil. The favorable fatty acids do not possess properties in common, such as melting point or length of chain. We do not understand why there should be such differences among the glycerides of these fatty acids.

When a small but inadequate amount of vitamin B (50 mg. of yeast) was fed, the results were entirely different, as can be seen by inspection of Fig. 2. The natural fat (cottonseed oil Diet 568) was definitely superior in its sparing action on vitamin B to

the glycerides of the individual fatty acids. No difference in the ability of the various glycerides to spare vitamin B was noticed. The growth of the animals receiving the stearin diet (Diet 600) was poorer than that obtained on the fat-free Diet 542. This difference we attribute to the poor utilization of stearin. The animals receiving the ethyl ester of palmitic acid (Diet 608) were in fairly good condition, though not so good as those receiving

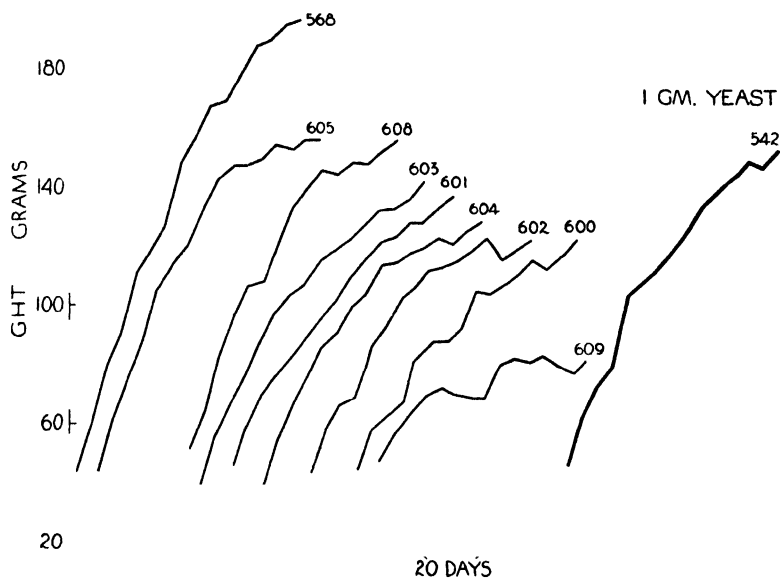


FIG. 3. Each curve represents the average growth obtained with four animals when 1 gm. of yeast was added to diets containing 25 per cent of the glycerides (see Table III for detail) in the order stated in the legend to Fig. 1. All the diets were supplemented with 2 drops of cod liver oil daily.

the glyceride of this acid. Inferior growth was obtained on the stearin diet (Diet 600), but the growth on the ethyl ester of stearic acid (Diet 609) was even poorer. The poor results obtained with stearin (Diet 600) may be explained on the basis of imperfect absorption, but it is not clear that this is due to its high melting point, for ethyl stearate (Diet 609), melting below the body temperature of the rat, is but slightly better absorbed than the stearin.

At the level of 1 gm. of yeast, the cottonseed oil was again superior to the other diets (Fig. 3). Now that vitamin B had been largely eliminated as a limiting factor there undoubtedly entered an unknown factor which made for the superior performance of animals on the natural fats and for inferior performance on the single fatty acid glycerides. Instead of conferring superiority, some fatty glycerides actually depressed growth below that obtained on a fat-free diet. The ethyl ester of palmitic acid (Diet 608) produced effects equal to those of the glyceride of caprylic acid (Diet 605). Growth with the glyceride of stearic acid (Diet 600) was rather poor, but with the ethyl ester of stearic acid (Diet 609) it was much poorer.

The diets containing the glycerides of caprylic, lauric, and myristic acids were fed to a second group of animals. The results were essentially the same as those obtained in the series reported. In the absence of vitamin B the diets containing the various glycerides produced results definitely superior to any obtained with natural fats, such as lard, coconut oil, and perilla oil. At the levels of 50 mg. of yeast and 1 gm. of yeast, the results were strictly comparable to those obtained in the first series.

#### SUMMARY

1. It has been previously shown that the liberal inclusion of fat in the diet will enable an animal to withstand for many weeks the withdrawal or omission of the antineuritic vitamin B, and in the presence of fat more growth will occur at any definite level of vitamin B than is the case with a fat-free diet.

2. In the absence of vitamin B some glycerides of single fatty acids permit better growth than do natural fats. The glycerides differ among themselves, those of myristin and caprylin being more effective than the others. But in the presence of vitamin B, natural fat is superior to any of the single glycerides.

3. The glyceride of stearic acid exerts no sparing action on vitamin B; in fact, the animals on this diet are in poorer condition than those on the fat-free diet. The poor growth performance of the stearin can perhaps be attributed to the fact that it is very poorly absorbed, its bulk in the food causing a partial starvation.

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## CRYSTALLINE VITAMIN D\*

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(Received for publication, February 11, 1932)

In a recent communication (1) we noted that the crystalline preparations of vitamin D reported by English and German workers had less antiricketic potency than certain non-crystalline preparations of our own. This observation has led to renewed and successful efforts to induce crystallization in the active resinous products already described.

The comparative evaluation of potency in the preparations from different laboratories has, until now, been practically impossible on account of the fact that bioassay technique differed widely in such essentials as duration of test, criterion of response, and definition of unit. In our critique of the line test (2) we have described means of translating these variables, and in the following paragraphs we have translated the findings of the leading investigators into the recently adopted international (League of Nations) units (3).

Our former basis of expressing potency was the "cod liver oil coefficient." The relation of this to the international unit (formerly the British unit) is centesimal, average cod liver oil having 100 international units per gm. This relation was determined directly by bioassay of the British standard solution, and reported in the "Critique." Several of the British workers have personally informed us that their assays on cod liver oils confirm our findings.

In 1928 we reported (4) that mercury arc radiations acting upon ergosterol in alcoholic solution in a simple quartz cell gave a product which, at the maximum of its activation curve, had a cod liver oil coefficient of 250,000. Spectrographic examination

\* Presented at the meeting of the American Association for the Advancement of Science at New Orleans, December 30, 1931.

showed that all but 27 per cent of this product was unchanged ergosterol. The non-ergosterol portion therefore had a potency of 93,000 international units per mg.:

$$250,000 \times 0.1 \times \frac{100}{27} = 92,593$$

(A)      (B)      (C)

where  $A$  = the reported cod liver oil coefficient,  $B$  = the factor to convert the cod liver oil coefficient to international units per mg., and  $C$  = the factor to allow for the unchanged ergosterol present.

We later reported (5) that the substitution of ether for alcohol in the above experiment greatly increased the potency. The crude product had a cod liver oil coefficient of 710,000, or 71,000 international units per mg.

In 1930 we attained (6) a cod liver oil coefficient of 1,000,000, equivalent to 100,000 international units per mg. This was accomplished by the more rigid exclusion of oxygen from the ether solution of the previous experiment. We noted that the product was not pure vitamin D, and that the absorption curves which various investigators had ascribed to the pure vitamin differed confusingly, one from another.

In 1931 we described (1) the apparatus and method devised for activating ergosterol in large amounts. The product was separated from unchanged ergosterol as a sticky resin having a cod liver oil coefficient of 250,000, or 25,000 international units per mg. We gave evidence, based upon changes in the heat of combustion after progressive oxidation, that this potent resin actually contained but a small amount of vitamin D.

By European workers, several crystalline preparations of vitamin D have been described. Jendrassik and Keményffi (7) in 1929 described their Fraction III-a as follows:  $[\alpha]_D = -35.4^\circ$  (in chloroform?); m.p.,  $92^\circ$ ;  $\lambda$ , none; daily dose for "völlige Heilung," 0.01  $\gamma$ . From private correspondence with Dr. Jendrassik we learn that "völlige Heilung" signifies approximately 2.4 + healing on the scale of our "Critique." This healing was obtained in 7 days. The potency of the crystals is thus calculated to be 90,000 international units per mg.:

$$17,000 \div (0.01 \times 0.82 \times 2.3) \times 0.1 = 90,138$$

(A)      (B)      (C)      (D)      (E)

where  $A$  = the daily dosage in  $\gamma$  of average cod liver oil required to give 2.0 + healing in 5 days,  $B$  = the reported daily dose in  $\gamma$  (Jendrassik and Keményffi),  $C$  = the factor to convert 2.4 + healing to 2.0 + healing,  $D$  = the factor to convert the 7 day test to the 5 day test, and  $E$  = the factor to convert the cod liver oil coefficient to international units per mg.

From the correspondence with Dr. Jendrassik it appears that his crystals, when assayed comparatively with the international standard, exhibit a potency of about 50,000 units per mg. The mean of this value and the value calculated above is 70,000 international units per mg., which one may consider a reasonable evaluation of Jendrassik's product.

Reerink and van Wijk (8) in 1931 described their latest crystalline preparation as follows:  $[\alpha]_D = +100^\circ$  (in ether); m.p.,  $117^\circ$ . Since no bioassays were given for this preparation, we must consider their earlier product (9) described in 1929: m.p.,  $< 0^\circ$ ;  $\lambda = 267 m\mu$ . Bioassays were made on a specimen calculated to contain about 50 per cent of vitamin D, the animal work having been done by van Niekerk and Everse (10). A dose of 0.01  $\gamma$  daily for 14 days gave a degree of healing which would be designated about 3.4 + on our scale (2). The potency of this specimen was therefore 69,000 international units per mg.:

$$\begin{array}{cccccc} 17,000 \div (0.01 \times 0.4 \times 6.12) \times 0.1 = 69,444 \\ (A) \quad (B) \quad (C) \quad (D) \quad (E) \end{array}$$

where  $A$  = the daily dosage in  $\gamma$  of average cod liver oil required to give 2.0 + healing in 5 days,  $B$  = the reported daily dose in  $\gamma$  (Reerink and van Wijk),  $C$  = the factor to convert 3.4 + healing to 2.0 + healing,  $D$  = the factor to convert the 14 day test to the 5 day test, and  $E$  = the factor to convert the cod liver oil coefficient to international units per mg.

Askew, *et al.* found that the crystalline "calciferol" described by them with Bourdillon in 1931 (11) was an isomorphous mixture of the vitamin with inactive material. Their most recent preparation (12) of supposedly pure crystals, separated via the 3,5-dinitrobenzoate, showed the following characteristics:  $[\alpha]_{5461}^{20} = +119.5^\circ$ ;  $[\alpha]_D^{20} = +105^\circ$  (in alcohol); m.p.,  $117^\circ$ ;  $\lambda$ , 265  $m\mu$ . The potency by direct assay against the international standard solution was 40,000 units per mg.

Windaus, Lüttringhaus, and Deppe (13) have lately crystallized



vitamin D after removing inert material with maleic or citraconic anhydride. They recognize two forms of the vitamin, D<sub>1</sub> and D<sub>2</sub>. The constants of vitamin D<sub>1</sub> were  $[\alpha]_{5461}^{20} = +171^\circ$ ;  $[\alpha]_D^{20} = +140.5^\circ$  (in acetone); m.p., 125°;  $\lambda = 265\text{ m}\mu$ . Windaus uses the assay method of Holtz, Laquer, Kreitmair, and Moll (14). Since this method is preventive, rather than curative, our regular translation factors are not applicable to it. However, we have used it in a comparison of assay techniques wherein test material was exchanged with Dr. Holtz and independently evaluated. Thus indirectly a translation is easily made. A certain preparation, H-92, was found to require a daily limit dose of 0.04  $\gamma$ . Its cod liver oil coefficient was 120,000, equivalent to 12,000 international units per mg. The potency of the present preparation, limit dose, 0.025  $\gamma$ , is thus calculated to be 19,000 international units per mg.:

$$\frac{0.025}{0.04} = \frac{12,000}{x}. \quad x = 19,200$$

Vitamin D<sub>2</sub> has very recently been described by Windaus, Linsert, Lüttringhaus, and Weidlich (15):  $[\alpha]_{5461}^{20} = +98.6^\circ$ ;  $[\alpha]_D^{20} = +82.6^\circ$  (in acetone); m.p., 116°;  $\lambda = 265\text{ m}\mu$ . The daily preventive limit dose was 0.015  $\gamma$ , which we translate, as above, to be equivalent to 32,000 international units per mg. Direct comparison against the international standard indicated a potency of about 40,000 units per mg. Taking the mean of these values, we estimate the potency of vitamin D<sub>2</sub> as 36,000 international units per mg.

The realization that our active resins were of the same order of potency as several crystalline preparations led us to consider that they should be crystallizable. The known tendency of impure sterols to remain vitreous when cooled below their melting points suggested that crystallization was but a matter of time. Accordingly, we examined several active resins which had been put aside and protected from decomposition. These resins were commercial batches stored at 0° in an atmosphere of CO<sub>2</sub>. They had been prepared by exposing ergosterol in ether solution to the radiations of a carbon arc, and subsequently removing the unchanged ergosterol by treatment with methyl alcohol (1). Their potency was 25,000 international units per mg.

Slow crystallization was observed to occur in material which

had stood for several weeks. At first only scattered islands of fine needles were seen; these gradually became rosettes which under magnification resembled chestnut burrs. The older preparations were solid crystalline masses. The photomicrographs in Figs. 1 and 2 show the crystals at early stages of development.

It is significant that this activated ergosterol preparation, known to be impure (1), went over completely into a crystalline mass merely on standing. If it had been even more impure, its melting point might well have been so depressed as to make crystallization impossible. The experiment demonstrates the marked tendency of ergosterol irradiation products to form mixed crystals. We



FIG. 1

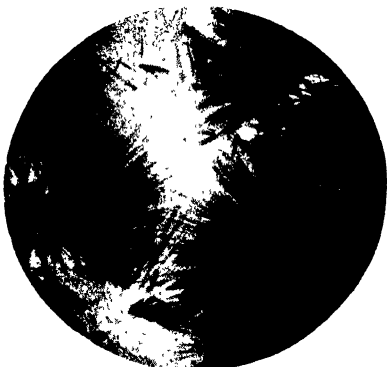


FIG. 2

FIGS. 1 AND 2. Early stages of crystallization of vitamin D resin. About 10  $\times$ .

maintain that the crystalline preparations of vitamin D thus far described should not be regarded as anything more than convenient starting material for further fractionation. One should not lose sight of the fact that the crystals obtained by Jendrassik and Keményffi showed no absorption maximum in the ultra-violet region. Our findings (1, 6) also indicate that greater purity of the vitamin will be indicated by decreased absorption.

#### SUMMARY

1. Translation into international units of the potencies reported for several crystalline preparations of vitamin D reveals wide variation in antiricketic value.

2. Under suitable conditions a resinous preparation can pass into the crystalline state without change of purity.

3. The crystalline state is evidence of relative, not absolute, purity in vitamin D. The best crystalline preparations as yet described are probably isomorphous mixtures containing a large percentage of inert material.

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## CALCIUM AND PHOSPHORUS STUDIES

### I. THE EFFECT OF CALCIUM AND PHOSPHORUS OF THE DIET ON TETANY, SERUM CALCIUM, AND FOOD INTAKE OF PARATHYROIDECTOMIZED RATS\*

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In the study of experimental hypoparathyroidism two factors frequently cause contradiction and confusion: (1) the anatomical relation of the parathyroid in the animals employed, and (2) the composition of the diet, especially with respect to its calcium and phosphorus content. In experiments with the dog or the monkey the extirpation of the parathyroids without destruction of thyroid tissue is frequently impossible and the complete operation of thyroparathyroidectomy must be used. That the thyroid alone has a profound influence on calcium and phosphorus metabolism has long been appreciated, and recently Aub and his associates (1) have demonstrated this fact by metabolism experiments in human beings. Hence, the removal of the thyroid with the parathyroids may cause an alteration in calcium and phosphorus metabolism different from that observed when either of them alone is removed. The rat, on the other hand, has but two encapsulated and easily removable parathyroids. Erdheim (2) and his followers made use of this anatomical fact and produced tetany in rats by cauterizing the parathyroids, and causing little or no destruction of the adjacent thyroid tissue. Erdheim showed that

\* Aided by a grant from Mead Johnson and Company, Evansville, Indiana. A preliminary report of the first part of this investigation appeared in *Proc. Soc. Exp. Biol. and Med.*, **23**, 301 (1930); the second part was read before the Eastern Society for Pediatric Research at New Haven, May 2, 1931.

parathyroidectomy in the rat produces faulty dentition, delays calcification of a callus, and impoverishes the body in lime salts.

The experiments of Erdheim were conducted prior to our more exact knowledge of complete diets and hence no control of calcium and phosphorus intake was considered. Even in recent times the diets used in experiments on dogs and monkeys have consisted mostly of meat and biscuit, and hence have been high in phosphorus and low in calcium. The addition of milk or calcium salts to such a diet is looked upon by some (3) as a departure from the usual feeding procedure; and milk is frequently referred to as having detoxifying properties in relieving parathyroid tetany (4). That the beneficial effects are due to the high calcium content of the milk must be apparent, and its mode of action has thus been explained by Salvesen (5). It is obvious that in the normal animal the parathyroids regulate the serum calcium level, even in the face of a very small intake. However, when this regulatory mechanism is removed, the small amounts of the calcium ingested are insufficient to maintain a normal serum calcium concentration, especially when the phosphorus concentration becomes elevated, and part of it must be either excreted or deposited as calcium salts.

With these facts in mind, the effect of the calcium and phosphorus content of the diet on the tetany and serum calcium has been studied in parathyroidectomized rats.

#### EXPERIMENTAL

The rats were of our own breeding stock, approximately 6 months of age and in perfect health. At least two rats were placed on each diet and the experiments were repeated several times at different periods, so that nearly 250 animals were used in all.

The parathyroids in the rat are easily accessible for surgical removal. There is only one on each side which is situated quite superficially in the thyroid substance and can be excised with little or no destruction of thyroid tissue. After the operation the animals were placed in wire mesh, raised bottom cages and kept in a ventilated room from which direct sunlight was excluded. In most instances the animals were placed on low calcium diets soon after operation and were kept on the diet until tetany de-

veloped, at which time they were changed to the respective experimental rations. Food and distilled water were allowed *ad libitum*. The composition of the diets is given in Table I.

The major part of the experiment was concerned with the effect of diets low in calcium and the same diets to which calcium was added, but some experiments in which either the calcium or the

TABLE I  
*Composition and Percentage of Calcium and Phosphorus of Different Diets Used*

<i>Diet I-K</i>		<i>Diet 33-A</i>	
Whole wheat flour .....	40.0	Diet I-K + 6.0 gm. per cent anhy-	
Wheat gluten .....	5.0	drous $\text{Na}_2\text{HPO}_4$	
Purified casein .....	10.0	P = 1.780 gm. per cent	
Butter.. ..	5.0	Ca = 0.012 " " "	
Olive oil .....	4.0		
Salts (Ca-free).....	3.5		
Starch .....	32.5		
		<i>Diet 32-A</i>	
Ca = 0.012 gm. per cent		Diet 33-A + 1.0 gm. per cent $\text{CaCO}_3$	
P = 0.475 " " "		Ca = 0.412 gm. per cent	
		P = 1.780 " " "	
<i>Steenbock-Bills (6) Stock Diet</i>		<i>Steenbock Rachitogenic Diet (7)</i>	
Yellow corn .....	76.0	Yellow corn .....	76.0
Crude casein .....	5.0	Wheat gluten ....	20.0
Linseed oil meal.....	16.0	NaCl .....	1.0
Alfalfa meal.....	2.0	$\text{CaCO}_3$ .....	3.0
NaCl (iodized) .....	0.5		
$\text{CaCO}_3$ .....	0.5		
	100.0	Ca = 1.240 gm. per cent	
Whole milk powder .....	33.3	P = 0.250 " " "	
Ca = 0.515 gm. per cent			
P = 0.450 " " "			

phosphorus of the diets was raised above optimal levels are also included. With the exception of the Steenbock rachitogenic diet (7), the rations were "complete" save for variations in calcium and phosphorus.

### Results

*Effect of Low Calcium Diets*—The low calcium diet used in the earlier experiments was the Steenbock rachitogenic diet from which

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the added  $\text{CaCO}_3$  was omitted. It contained about 0.040 gm. per cent of calcium and 0.250 gm. per cent of phosphorus. In addition to its paucity in calcium, the protein of this ration is of an inferior grade. In subsequent experiments a low calcium diet (Diet I-K) was used in which the quality of protein was better but the calcium content was reduced to 0.012 gm. per cent.

In the early experiments rats were placed on the low calcium diet a few days prior to the operation in order to eliminate the absorption of residual fecal calcium. This procedure, however,

SERUM CALCIUM  
MMS.

14.

NORMAL STOCK DIET  
AND  
2K + 1.0 GM.  $\text{CaCO}_3$

LOW CALCIUM DIETS

OPTIMAL CALCIUM-  
HIGH PHOSPHORUS

DAYS OF  
DIET 30

FIG. 1. Concentrations of serum calcium of parathyroidectomized rats on varying calcium- and phosphorus-containing diets at different periods after operation.

was found to be hazardous to the lives of the animals, as most of them developed severe tetany and died in status tetanicus within 12 to 24 hours after operation. For this reason, the rats used in the subsequent experiments were kept on the stock diet until the operation and then placed on the low calcium diet. This reduced the mortality to a minimum, and the tetanic symptoms developed more gradually, usually not for 36 to 48 hours or even longer. The serum calcium levels of parathyroidectomized rats kept on

this diet for varying periods of time are given in Fig. 1. The serum calcium levels ranged between 7.2 and 2.5 mg. per cent and paralleled the severity of the tetany fairly closely. The serum inorganic phosphorus concentrations varied from 4.7 to 20.8 mg. per cent and there was no definite inverse relation to the serum calcium concentration. The inorganic phosphorus values, how-

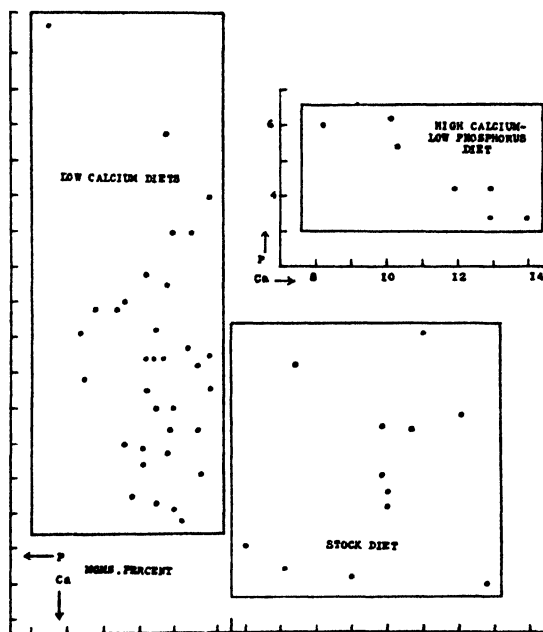


FIG. 2. The concentrations of serum inorganic phosphorus of parathyroidectomized rats on varying concentrations of calcium and phosphorus of the diets and at different serum calcium levels. The concentrations of the serum inorganic phosphorus of unoperated middle-aged rats on the same diets range between 2 to 5 mg. per cent.

ever, tended to be higher, the graver the tetany and the lower the serum calcium (Fig. 2).

*Effect of Optimal Calcium and Phosphorus Diets*—In this group two diets were employed: (1) the Steenbock-Bills stock diet, and (2) the low calcium diet (Diet I-K) to which 1.0 gm. per cent of  $\text{CaCO}_3$  was added. Their compositions are given in Table I.



They are complete with respect to protein, mineral, and vitamin requirements. Their calcium and phosphorus contents differ somewhat; the former contains 0.515 gm. per cent of calcium and 0.450 gm. per cent of phosphorus and the latter 0.412 gm. per cent of calcium and 0.475 gm. per cent of phosphorus. The response to calcium-containing diets, after the animals developed tetany on a low calcium diet, was variable and sometimes rather slow. While some remained in a state of tetany, the majority of the animals lost their tetanic manifestations and the serum calcium concentration rose to normal or slightly subnormal levels. The tendency for the serum inorganic phosphorus was to be lower in this group than in the low calcium group but it was higher than normal even when the serum calcium concentration returned to non-tetanic levels (Fig. 2).

*Effect of Steenbock Rachitogenic Diet*—The two significant chemical changes in the blood after parathyroidectomy are: (1) the retention of phosphorus and its elevation in the serum, and (2) a reduction in the serum calcium. The rachitogenic diet which is high in calcium and low in phosphorus seemed to be the most efficacious in relieving tetany and raising the serum calcium levels. Although serum calcium determinations were made on only seven animals of this group, experience with this diet in other parathyroidectomized animals which were not bled or sacrificed indicated that the animals ate optimal amounts of this diet, and that it alleviated their tetanic symptoms. The serum calcium concentrations in this group ranged between 8.2 and 14.0 mg. per cent. The inorganic phosphorus was lower than in any other dietary group and ranged between 3.4 and 6.2 mg. per cent.

*Effect of Low Calcium-High Phosphorus Diet*—This diet is faulty in two respects. Not only is its calcium content very low but its phosphorus concentration is excessively high. Normal animals fed such a diet may develop osteoporosis and low serum calcium concentration. Parathyroidectomized animals fared badly on this diet, and most of them went into a state of continuous tetany, lost weight, became emaciated, and finally died. Only two survived 60 days and the concentration of the serum calcium of their pooled blood was 3.6 and that of phosphorus 12.3 mg. per cent. The reason for the loss of weight and rapid decline was not entirely clear at first, but later metabolism experiments with

high phosphorus diets indicated that the animals may have eaten very little, if at all.

*Effect of Minimal Calcium-High Phosphorus Diets*—The ration used in this group was the low calcium-high phosphorus diet employed in the preceding experiment, except that 1.0 gm. per cent of  $\text{CaCO}_3$  was added to it. The food consumption in this group was slightly better than in the preceding group, but it was still decidedly low. The rats were undernourished as compared to similar experimental animals kept on optimal or high calcium-low phosphorus diets. Two animals remained in this undernourished state for 6 months. Their combined serum calcium concentration was 5.4 mg. per cent.

*Effect of Alternation of Diets*—A series of animals was first kept on the low calcium diet for a few days after operation, and as soon as severe tetany developed 1.0 gm. per cent of  $\text{CaCO}_3$  was added to the diet. The response to this amount of calcium addition was rather slow, and sometimes about 20 to 25 days elapsed before the animals relaxed entirely. On the other hand, when later the calcium addition was omitted from the diet, severe tetany ensued within 5 days or sooner; i.e., the induction of tetany in relaxed animals is accomplished more rapidly by removal of calcium than its alleviation by the addition of the same amount of calcium to the diet. It is quite possible that 0.412 gm. per cent of calcium, which the diet contained, barely suffices to keep a parathyroprivic animal in calcium equilibrium and, therefore, either the calcium intake has to be increased or the phosphorus consumption sufficiently lowered, in order to effect a more rapid cure. For this reason the high calcium-low phosphorus diet proved to be more efficacious than either the stock diet or the low calcium diet to which 1.0 gm. per cent of  $\text{CaCO}_3$  was added.

*General Effects*—Changes in ectodermal structure in idiopathic tetany have been described by many observers. Cataracts, brittleness and ridging of nails, loss of hair, and defects in the enamel of the teeth are the most common changes reported. The most common changes observed in over 200 operated rats were: (1) loss of nails and bleeding from the nail beds, (2) scattered areas of alopecia, (3) changes in the tooth structure. The latter consisted in hyperplasia and curving of the incisors. Frequently molar caries was found. Gross cataracts were never seen. It is

possible, though, that the absence of cataracts may have been due to the relatively short experimental periods which were insufficient for the maturation of lenticular opacities; or the exclusion of direct sunshine may have prevented their occurrence.

*Effect of Diet and Parathyroidectomy on Weight and Food Intake*—The loss of weight and emaciation seen in thyroparathyroidectomized dogs is usually attributed directly to loss of thyroid function and the survival of operated dogs on diets to which milk has been added is attributed, not to the calcium intake, but rather to functioning parathyroid rests. In rats parathyroid rests are very uncommon and in our rats the thyroids were not removed. The condition of the animals, however, was definitely influenced by the diet. It was found that operated rats kept on diets low in calcium or excessively high in phosphorus lost weight and continued to have tetany; whereas, those fed the stock diet or a high calcium-low phosphorus diet gained or maintained their weights. In most instances the severity of the tetany was proportional to the weight changes. This was not only true of animals kept on the given diets throughout the experimental periods, but it was also observed when the diets were alternated. The same animals lost weight when diets gave rise to tetany and gained weight on calcium-containing diets which relieved the tetanic symptoms.

That the calcium and phosphorus content of the diet rather than the operative procedure was responsible for the anorexia is illustrated by the following experiment. Twelve adult rats were parathyroidectomized in the same forenoon. One of the animals (Rat 1434) bled during the operation and, consequently, uncertainty existed as to the extirpation of both parathyroids. After operation the animals were placed on a low calcium diet. Rat 1434 failed to develop tetany and maintained its weight, while the other eleven developed tetany and lost weight quite promptly. All the animals were bled by cardiac puncture 7 days after operation and the serum calcium concentrations of the eleven were at tetanic levels while that of Rat 1434 was 9.3 mg. per cent. One group of three of these animals was then placed on the Steenbock rachitogenic diet, another on the stock diet, and a third group, including Rat 1434, was given the low calcium diet. The diets were fed for 16 days and the animals were then killed. The results are indicated in Table II. It is seen that Rat 1434 con-

tinued to gain weight and had only slight evidence of tetany and a serum calcium concentration of 7.6 mg. per cent in spite of being fed the low calcium diet for more than 3 weeks. Its group mates continued to have tetany and lost considerable weight. One of them died 19 days after operation in status tetanicus; the other, at the time of killing, had tetany and concentrations of serum calcium and inorganic phosphorus of 5.9 and 9.4 mg. per cent,

TABLE II

*Weights and Serum Calcium and Inorganic Phosphorus Concentrations of Parathyroidectomized Rats before and after Change of Diets*

	Rat No.	Change of diets			Weight			Remarks
		Before	After		Before operation	End of low Ca diet	After change of diet	
		Ca	Ca	P				
		mg. per cent	mg. per cent	mg per cent	gm.	gm.	gm.	
Group 1, Steenbock rachitogenic diet	1426	4.3	12.9	3.4	325	264	310	No tetany
	1427	4.5	10.3	5.4	260	218	255	" "
	1428	4.9	8.2	6.0	290	245	281	" "
Group 2, stock diet	1429	5.9	10.9	9.4	315	250	325	" "
	1430	5.9	9.9	9.5	312	247	320	" "
	1431	6.5	11.0	12.1	305	240	308	" "
Group 3, low Ca diet continued	1432	4.2			250	218	162	Died in status tetanicus
	1433	5.0	5.9	9.4	305	255	190	Severe tetany
	1434	9.3	7.6	5.6	318	306	322	Very mild tetany, only one parathyroid removed

respectively. The two trios receiving the stock diet and the rachitogenic diet regained their weights quite promptly. The alleviation of the tetany was more pronounced on the rachitogenic diet, although the gain in weight was better on the stock ration, probably due to the superiority of the protein in the latter ration. At autopsy it was found that the parathyroids were removed completely from all the animals except Rat 1434, in which an intact

left parathyroid was discovered. The concentrations of serum calcium and inorganic phosphorus of these animals are shown in Table II.

When these observations in regard to the relation of the calcium and phosphorus of the diet to weight and tetany were subjected to an analysis, it was found that gains and losses in weight were due to variations in the amount of food consumed. The greatest loss of weight and the most pronounced tetany occurred in the groups of rats fed either low calcium-optimal phosphorus or high phosphorus-optimal calcium diets. The weight loss was due, appar-

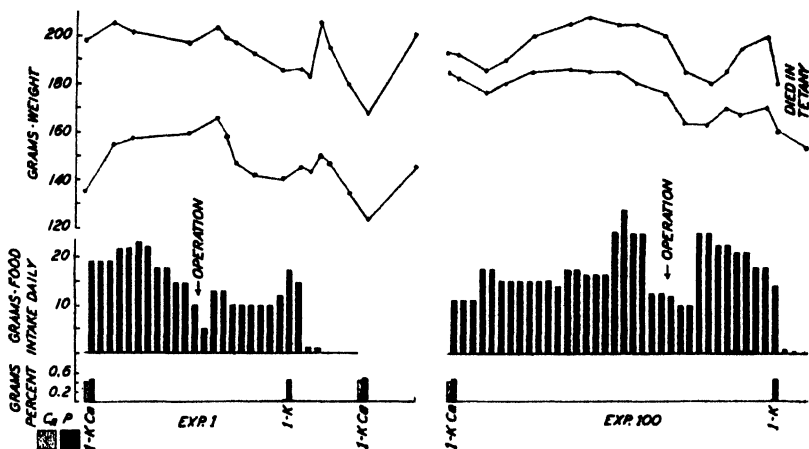


FIG. 3. Effect of low calcium diet on the daily food intake and the weight curves of parathyroidectomized rats. The percentage composition of the food calcium and phosphorus is also shown.

ently, to an accompanying anorexia. This observation suggested that the excess phosphorus in the diet might be responsible, in part, for the anorexia and the continuance of the tetanic symptoms. To test the correctness of this hypothesis, a quantitative study was made of the food consumption of parathyroidectomized rats receiving diets varying in their calcium and phosphorus content.

Usually two healthy, young adult rats were used for each experiment. They were kept in a Hopkins-Gamble metabolism cage and the food intake was determined either daily or every few days. Distilled water was allowed *ad libitum*. The urine and

feces were collected separately for each period and analyzed for calcium and phosphorus. The animals were weighed at the same time as the food consumption was measured.

In the first experiment two rats were fed Diet I-K + 1.0 gm. per cent of  $\text{CaCO}_3$  for 12 days before operation and for 10 days afterwards. The diet was "complete" and contained 0.412 gm. per cent of calcium and 0.475 gm. per cent of phosphorus. On the 11th day after operation the animals were fed the calcium-poor diet, Diet I-K. The quantitative daily food consumption and the weights of the animals are given in Fig. 3 (Experiment 1). It is seen that the animals ate optimal amounts of the calcium-containing diet before and after operation, but as soon as most of the calcium was removed from the diet, anorexia and loss of weight developed within a few days. The animals ate the low calcium diet well for the first 2 days, but together consumed only 2 gm. in the next 2 days and none at all in the following 3 days. They lost nearly 20 per cent of their body weight during this period. When the calcium was replaced in the diet, the anorexia disappeared and the animals regained their weight within 5 days. The food intake was not measured during this last period.

In the second experiment a similar experience was encountered. The operated rats ate the calcium-containing diet, but refused to eat the diet from which the calcium was omitted. One of the animals died in status tetanicus 3 days after the change of diet, and the other had such pronounced tetany that it was necessary to place it on the stock diet. The results are represented graphically in Fig. 3 (Experiment 100).

In the third experiment three rats were used. They were kept on the calcium-containing diet, Diet I-K + 1.0 gm. per cent of  $\text{CaCO}_3$ , for 3 days prior to the operation and for 10 days afterwards. They were then changed to the low calcium diet, Diet I-K. The results were similar to those cited above. The operated animals ate the calcium-containing diet but refused the calcium-poor ration and after 4 days had severe tetany and lost weight. The diet was next changed so that the calcium was restored to its previous level and the phosphorus was increased to 1.780 gm. per cent by the addition of anhydrous  $\text{Na}_2\text{HPO}_4$ . On this ration the animals developed an extreme anorexia, most pronounced tetany, and cachexia. After 5 days the excess phosphorus

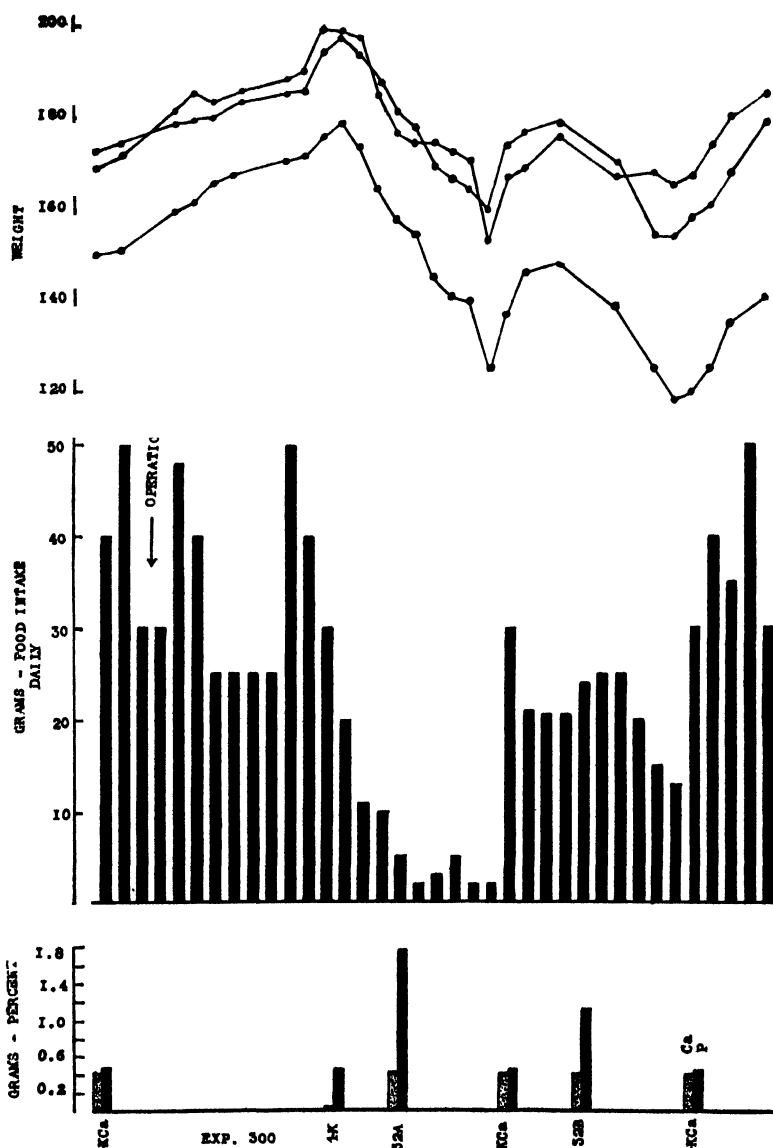


FIG. 4. Effects of low calcium diet, optimal diet, and the latter diet to which  $\text{Na}_2\text{HPO}_4$  was added, on the daily food intake and on the weights of parathyroidectomized rats.

was omitted from the diet but the calcium was retained. On this diet the anorexia disappeared quite promptly and the animals gained weight. When next the phosphorus of the diet was increased, this time to 1.130 instead of 1.780 gm. per cent, as previously, and the calcium held at 0.412 gm. (Diet 32-B), the food consumption increased but was still considerably lower than the intake of the same diets when the calcium and phosphorus were

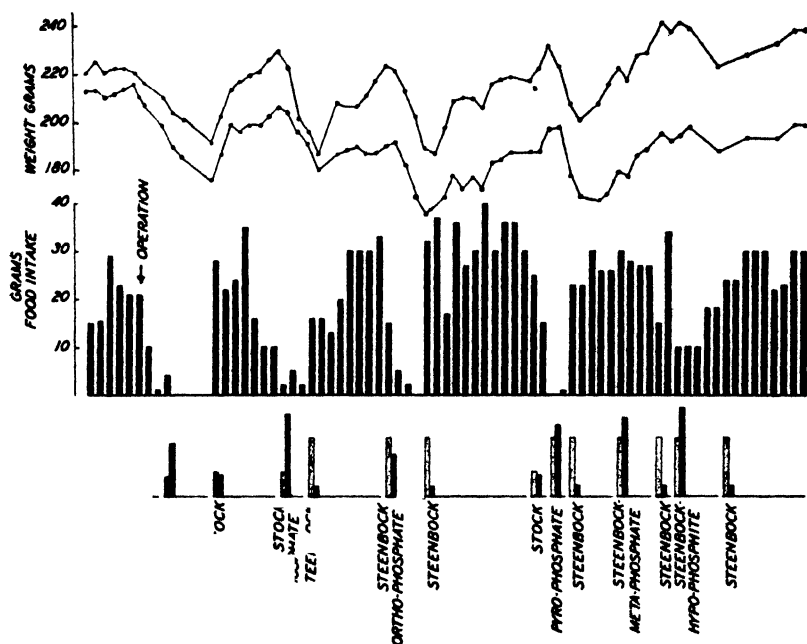


FIG. 5. Effects of variations in the calcium and phosphorus of the diet and of the type of phosphorus salt on the daily food intake and weights of parathyroidectomized rats.

optimal, as at the beginning of the experiment. When finally the added phosphorus was again removed from the diet, the animals again rallied, ate better, and gained weight. The results are shown in Fig. 4 (Experiment 300).

The foregoing three experiments seemed to indicate that the excess phosphorus over calcium in the diet determined the anorexia and the cachexia in the parathyroidectomized rats. These symp-



toms occurred when the diet was optimal in phosphorus but low in calcium, or when it was either optimal or low in calcium but high in phosphorus. Since in the above experiments no data were obtained on the intake of high phosphorus diets by normal rats, the experiment was repeated and the animals were fed the high phosphorus diet prior to as well as after parathyroidectomy. The results are indicated in Fig. 5. It is seen that, whereas the animals ate the high phosphorus diet before the operation, they refused to eat it afterwards.

After 3 days of anorexia part of the added phosphorus was omitted from the diet but the diet was still much higher in phosphorus than in calcium. The rats ate 4 gm. on the 1st day but declined to eat on the 2nd. They were then placed on the stock diet. At first they were suspicious even of this diet and refused to touch it for about 3 hours after it was placed in the feeding cup; but as soon as one of the animals tasted the ration and continued to eat it, the other animal made vigorous efforts to get to the food by biting and pulling its mate out of the cup. They together consumed 27 gm. of the diet on that day and optimal amounts for the following 6 days. They were then placed on the same stock diet to which 6.0 gm. of anhydrous  $\text{Na}_2\text{HPO}_4$  were added. They ate 10.0 gm. on the 1st day but very little for the next 3 days.

From these results it became increasingly evident that parathyroidectomized animals cannot tolerate an excess of phosphorus in their food. Analyses of the inorganic phosphorus of the serum and of the excreta have shown (8) that parathyroidectomized animals retain more phosphorus than do normal animals. Since the lowering of the serum calcium concentration may be the result of the phosphorus retention it was anticipated that the feeding of a high calcium-low phosphorus diet would be beneficial to the animals. The Steenbock rachitogenic diet (7), which contains about 1.240 gm. per cent of calcium and 0.250 gm. per cent of phosphorus, fulfilled this requirement. On this diet the anorexia and the symptoms of tetany disappeared quite rapidly. When an excess of phosphorus was added to this diet, the animals again developed an anorexia and refused to eat, in spite of the fact that the ration was relatively high in calcium. They resumed eating the diet as soon as the added phosphorus was omitted.

Although the evidence, so far, pointed to the excess phosphorus

in the diet as the offending substance in the causation of anorexia in the operated rats, further proof was obtained from the following experiments. At the suggestion of Dr. E. V. McCollum, the following sodium salts of phosphorus were tried instead of the orthophosphate: (1) pyrophosphate,  $\text{Na}_4\text{P}_2\text{O}_7$ ; (2) metaphosphate,  $\text{NaPO}_3$ ; and (3) hypophosphite,  $\text{NaH}_2\text{PO}_2 \cdot \text{H}_2\text{O}$ . Since it is alleged that these salts are not utilized in the animal organism,

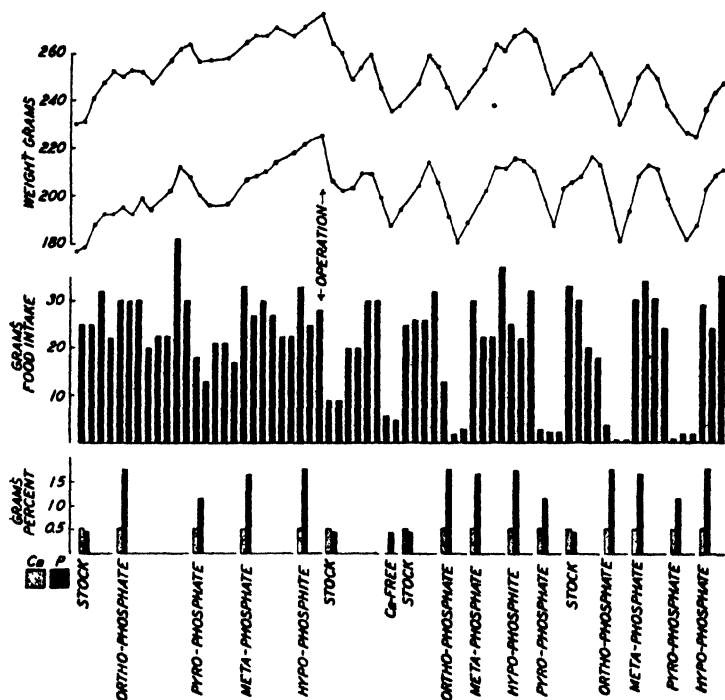


FIG. 6. Effects of addition of sodium-orthophosphate, metaphosphate, pyrophosphate, and hypophosphite on the daily food intake and growth curves of rats before and after parathyroidectomy.

the ingestion of an excess of these salts should not affect the food intake as do the orthophosphates. Fig. 5 shows the percentage of calcium and of phosphorus in the diets, the daily food consumption, and the weight curves. It is seen that the addition of sodium metaphosphate or hypophosphite to the Steenbock diet produced no detectable effects on the food intake or well being; whereas,

the addition of sodium pyrophosphate to the ration produced changes similar to those observed when the orthophosphate was fed. Variations in the effects of these salts may be due to the fact that the metaphosphates and hypophosphites are inert biologically, and they were either not absorbed from the gastrointestinal tract or were excreted unchanged in the urine and feces.<sup>1</sup>

In the fifth experiment the different phosphorus salts were fed to the animals before as well as after the removal of the parathyroids. The results are similar in all respects to those obtained in the above experiment. They are shown graphically in Fig. 6.

#### DISCUSSION

From the above results it would seem that in the study of parathyroid tetany the calcium and phosphorus of the diet are determining factors for the development or non-development of tetanic symptoms. The many conflicting conclusions in the literature concerning the cause of alleviation of parathyroprivic tetany may thus be explained by variations in the calcium and phosphorus intake in the various experiments. It would appear that not only is the absolute amount of dietary calcium of paramount importance but that the phosphorus content of the diet is also of importance, since it determines whether or not a given amount of calcium is efficacious in relieving tetany. With low or normal dietary phosphorus, a minimal amount of calcium may suffice to bring about relief from tetany, but, if the phosphorus is increased beyond physiological limits, the same amount of calcium fails to ameliorate the tetanic symptoms and to raise the calcium level in the blood. Thus the ability of milk supplements to relieve tetany in some instances and its failure in others may be due to the calcium and phosphorus contents of the other foodstuffs. Since milk contains nearly as much phosphorus as it does calcium, if it be added to a high phosphorus diet, such as meat, it may fail to relieve tetany because of the relatively high intake of phosphorus as compared to calcium; but if milk constitutes the sole dietary of the animal or the other food supplements be either low in phos-

<sup>1</sup> For a discussion on the physiological effects of these phosphorus salts in nutrition see Forbes and Keith (9).

phorus or high in calcium, the calcium ingested with the milk may suffice to relieve tetany and preserve the life of the animal.

The observations of Compere and Luckhardt (10) and of Inouye (11) are interesting in this connection. The two former have demonstrated the ineffectiveness of phosphate of calcium as against the carbonate, nitrate, and acetate in relieving tetany in parathyroprivic dogs. The latter has shown that lactose-containing diets which were able to control parathyroid tetany became ineffectual when casein was also included. His basal diets contained bone ash ( $\text{Ca}_3(\text{PO}_4)_2$ ) and a salt mixture in which calcium and phosphorus were present. The addition of a phosphoprotein, such as casein, merely increased the phosphorus intake and neither the amount of calcium in the diet nor the lactose afforded the animal protection from tetany.

One of the most constant findings in severe parathyroid tetany is the retention of inorganic phosphorus and its increase in the blood. Greenwald (8, 12) has suggested, on several occasions, that the disturbance in phosphorus metabolism in parathyroid deficiency is equally, if not more, important than the calcium disturbance. When the food calcium is minimal, the disturbance is reflected in a decrease of phosphorus excretion and at times by its entire absence from the urine. When calcium is added to the diet, the excretion of phosphorus increases and the severity of the tetany is diminished, but even under this condition the serum phosphorus may remain higher than normal (Fig. 2). This would seem to indicate that, whereas an animal with normal parathyroid function excretes its excess of ingested phosphorus regardless of the calcium intake, a parathyroprivic animal is unable to do so unless a sufficient amount of calcium be present in the diet and hence in the circulatory fluids. Thus, the increase of dietary calcium on an optimal or minimal phosphorus intake results in an inactivation of the retained phosphorus and in an elevation of the serum calcium concentration. When, however, the phosphorus intake is very high, the same amount of calcium is without avail in raising the serum calcium concentration. The results become more complicated by the fact that when the animals already have phosphorus retention they eat only very small amounts of phosphorus-rich food and thus ingest only small amounts of calcium as compared to phosphorus.

No definite explanation can be offered at present as to the physiologic mechanism responsible for the anorexia of parathyroidectomized rats fed tetany-inducing diets. On observing the animals during the period of anorexia it was found that, if the diet was conducive to tetany, they partook of a small amount of the food at first and then suddenly stopped eating. On the other hand, if after a period of anorexia the animals were placed on an optimal dietary régime, they were suspicious at first, but having once ventured to taste the diet and, finding it inoffensive, they continued to eat it. Apparently there is a direct relationship between an excess of a substance in the diet of which the animal will partake and the degree of its retention in the organism. Since phosphorus is the substance retained after parathyroidectomy, it is not at all surprising that when its concentration in the body reaches a certain level, the animals will refuse diets which tend to intensify its retention. This is borne out by the fact that, when the diet is changed from an optimal one to one producing tetany, the anorexia may come on gradually, as the retention of phosphorus increases.

That the excess dietary phosphorus and the phosphorus retention are responsible for the anorexia is further substantiated by its presence only when the dietary phosphorus is utilized and its absence when the phosphorus salts are either not absorbed or are excreted unchanged.

It seems possible that the anorexia in parathyroidectomized rats is analogous to the vomiting in parathyroprivic dogs fed meat diets. "Rodents," according to Hatcher (13) "have probably lost the capacity to vomit through the development of a better means of protection against poison," while dogs apparently resort to anorexia and also vomiting as means for protection. If, after parathyroidectomy, the organism is unable to rid itself of its retained phosphorus unless a sufficient amount of calcium is ingested, then the feeding of diets which augment the phosphorus retention might provoke vomiting or anorexia. That the mechanism *may be* neurogenic is suggested from the fact that the animals taste the food first and then stop eating. Apparently, as in Hatcher's (13) experiments with digitalis vomiting in dogs, the offending substance must come in contact with the nerve endings in the gastric mucosa, from which impulses are trans-

mitted to Thumas' area (13). The reflex impulses emanating from this area cause either vomiting or, in the rat, its equivalent, anorexia.

In the light of this discussion the supposition that meat contains a toxic substance conducive to tetany and that milk is endowed with a parathyroid hormone or a detoxifying substance (4) becomes superfluous. The susceptibility to tetany and its cure depend largely upon the calcium and phosphorus content of these two food substances, as has been previously pointed out by Salvesen (5).

The explanation of divergent results in parathyroidectomy experiments on the basis of functioning parathyroid rests (8) is hardly necessary, since dietary differences alone may account for the variations in experimental results.

#### SUMMARY AND CONCLUSIONS

1. The calcium and phosphorus content of the diet is a determining factor in the presence or absence of tetany in parathyroidectomized rats.

2. Low calcium or high phosphorus diets are conducive to parathyroprivic tetany; while high calcium or low phosphorus diets are ameliorative.

3. The serum calcium concentrations vary with the severity of the tetany and with the calcium and phosphorus intake.

4. Cachexia parathyroprivia was found to depend upon the food intake. Parathyroidectomized rats refuse to eat tetany-inducing diets and thus lose weight, but they consume optimal amounts of foods which relieve or prevent their tetanic manifestations.

5. The anorexia was present when dietary phosphorus was in a form which could be utilized by the animal organism but absent when the phosphorus salts could not be utilized. Sodium metaphosphate and hypophosphite belong to the latter group.

6. An analogy is drawn between the anorexia of parathyroidectomized rats on tetany-inducing diets and the vomiting of parathyroprivic dogs on similar rations.

7. Attention is called to the importance of phosphorus as a factor in the study of parathyroid tetany.

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## CALCIUM AND PHOSPHORUS STUDIES

### II. THE EFFECT OF DIET AND OF VIOSTEROL ON THE TETANY AND ON THE SERUM CALCIUM OF PARATHYROIDECTOMIZED RATS\*

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The mechanism by which viosterol is capable of raising the serum calcium and the inorganic phosphorus levels in the normal, rachitic, or tetanic organism is not as yet well understood. Since the levels of these substances in the blood of the normal animal depend, to a large extent, on the regulatory activity of the parathyroids, it has been suggested (1) that the hypercalcemia produced by viosterol is through its influence on these glands. This view was based on the observations of Hess and his coworkers (2). They have shown that the hypocalcemias produced in dogs and monkeys by feeding low calcium diets can be converted into hypercalcemias by means of large doses of irradiated ergosterol. After thyroparathyroidectomy, however, corresponding doses of ergosterol failed to raise the serum calcium above tetanic levels. Greenwald and Gross (3) report similar observations with the use of cod liver oil and viosterol,

The results of these investigators are at variance with those of Jones (4), Brougher (5), and of Demole and Christ (6), who were able to raise the serum calcium levels and to ameliorate the tetanic symptoms in parathyropivic animals by the administration of antirachitic agents. Wade (7), Urechia and Popoviciu (8), and Comel (9) have reported similar experiences.

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The discrepancies in the results of the different investigators may be attributed to: (1) the differences in the composition of the diets with respect to calcium and phosphorus, (2) the differences in the concentrations of the serum calcium before operation, and (3) variations in the viosterol or cod liver oil dosages. The diets used in the experiments of the aforementioned investigators are either not stated or seem to have been of an inconstant composition with regard to their calcium and phosphorus. The composition of the diet used by Hess is not given in detail—it was stated to be low in calcium—but from its ability to induce latent tetany and hypocalcemia, it may be inferred that it was high in phosphorus as well as low in calcium. The diets used by Greenwald and Gross were high in phosphorus. Greenwald and Gross obtained a beneficial result from vitamin D in one of their animals and attributed it to the chance admixture of a small amount of calcium in the infusorial earth used in the metabolism experiment; they explained the positive results in Brougher's and in Wade's experiments as due to the administration of milk or to the presence of parathyroid rests.

In Paper I (10) the importance of the relation of the calcium and phosphorus of the diet to the levels of these elements in the blood and to the tetanic symptoms was stressed. The present experiments deal with the effect of diet and of viosterol on the symptoms of tetany and on the concentrations of serum calcium and inorganic phosphorus in parathyroidectomized rats. The compositions of the diets and the conduct of the experiments are described elsewhere (10). In most instances, the viosterol was mixed in the diet. The dosage was expressed in terms of cod liver oil equivalents as percentage of the diet. Thus, when 1.0 cc. of viosterol 100 D was added to 100 gm. of diet, its vitamin D content was equal to 100 cc. of cod liver oil, or 100 per cent of the diet. Since the therapeutic dose of cod liver oil is about 0.25 per cent of the diet, 1.0 cc. of viosterol 100 D in 100 gm. of diet is equivalent to 400 times overdosage. The dosage of viosterol used in these experiments varied from 5 to 20,000 per cent<sup>1</sup> of the diet or 20 to 80,000 times the therapeutic dose.

<sup>1</sup> Viosterol 10,000 D, or its dilutions in oil were used whenever the dosage was higher than 500 per cent.

## Results

*Effect of Viosterol and Low Calcium Diet, Diet I-K*—This diet contained only 0.012 gm. per cent of calcium but was otherwise adequate. When 5 per cent viosterol was added to the diet and fed for 20 to 90 days to parathyroidectomized rats, in which tetany was first established, the serum calcium levels varied between tetanic levels and those found in latent tetany, *i.e.* 6.3 to 8.9 mg. per cent (Fig. 1). The severity of the tetanic symptoms paralleled the calcium levels. The two extreme values were, however, somewhat higher than in the animals fed the same diet without viosterol.

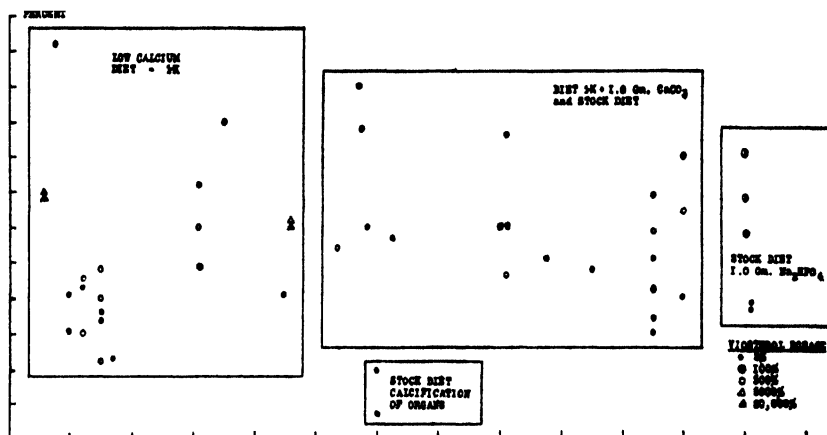


FIG. 1. The serum calcium levels of parathyroidectomized rats fed diets varying in their calcium, phosphorus, and viosterol content for periods of time indicated.

When the viosterol dosage was increased 100-fold, the serum calcium concentrations varied from tetanic to hypercalcemic levels. All the animals exhibited severe tetany before the viosterol feeding was begun, and hence the disappearance of the symptoms and the elevation of the serum calcium could only be attributed to the larger doses of viosterol. The concentration of serum calcium ranged between 6.2 and 13.0 and, in one instance, it was as high as 15.2 mg. per cent. The tetany followed the calcium levels in

the serum. Tetany and low serum calcium concentration were frequent among the animals failing to gain or losing weight, probably occasioned by a small intake of both food and viosterol. Those that gained weight and apparently partook of the viosterol-containing diet, soon lost their tetanic symptoms and their serum calcium concentrations rose to normal or even hypercalcemic

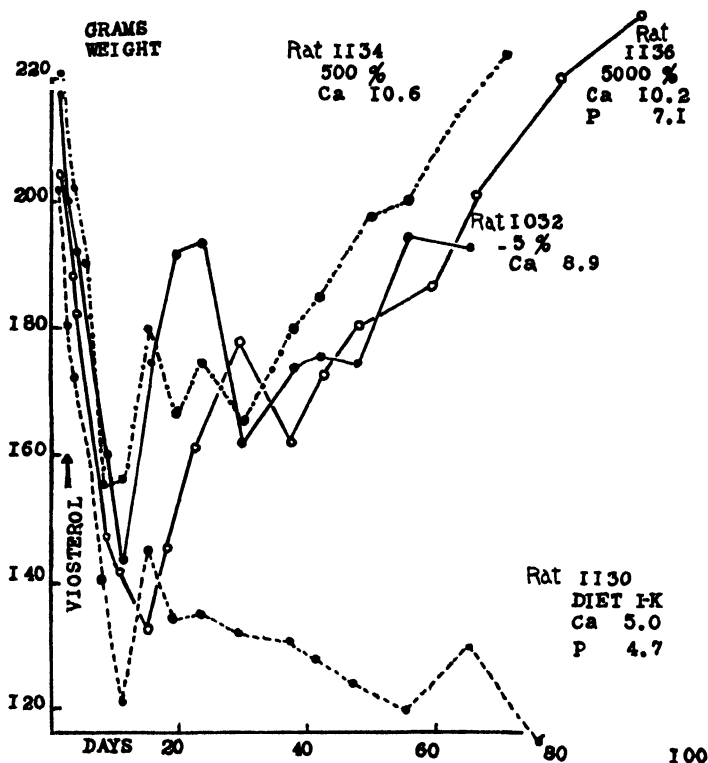


FIG. 2. Effect of viosterol on the weights and on the serum calcium levels of parathyroidectomized rats kept on a low calcium diet.

levels (Fig. 2). Thus, the lack of response to viosterol in some animals and the favorable influence in others may have been due, in part, to the differences in viosterol intake.

Large doses of viosterol were used in only a few animals. One rat, after having severe tetany, was placed on the low calcium diet with 5000 per cent viosterol (20,000 times overdosage) and kept

on this diet for 92 days. Before being killed, tetany was entirely absent and the concentration of serum calcium was 10.2 and that of inorganic phosphorus 7.1 mg. per cent. Two animals were similarly placed on the same diet with 20,000 per cent viosterol for 11 days. They were free of tetany at the end of this period and the concentrations of serum calcium and inorganic phosphorus in the combined blood were 11.0 and 9.6 mg. per cent, respectively.

Fig. 2 illustrates the effect of viosterol on the weight curves and the levels of serum calcium in one experiment. It is seen that the animal receiving no viosterol and continuing to have tetany failed to gain weight and had a low serum calcium concentration; whereas, those receiving viosterol and losing their tetanic symptoms gained weight and had normal serum calcium concentrations. Fig. 2 also shows that the response of parathyroprivic animals to viosterol does not become apparent until the antirachitic agent has been administered for some time. Thus, the animals continued to have tetany and to lose weight for about 10 days after viosterol administration had been begun, but as soon as they started to gain weight the tetanic symptoms began to disappear.

*Effect of Viosterol and Minimal<sup>2</sup> Calcium and Phosphorus Diet (Low Calcium Diet plus 1.0 Gm. Per Cent  $\text{CaCO}_3$ )*—This diet alone without viosterol raised the serum calcium in the parathyroidectomized rats rather slowly and irregularly from extremely low tetanic levels to low normal values. When 5 per cent viosterol was added, one rat, killed after 15 days, had no tetany and his serum calcium concentration was 12.8 mg. per cent. Another, observed for 90 days, remained free of tetany and, when killed, the concentration of serum calcium was 8.8 and that of inorganic phosphorus 4.8 mg. per cent.

When the dosage of viosterol was increased to 500 per cent, the tetanic symptoms disappeared quite rapidly and the serum calcium concentrations rose to levels above those of tetany except in the case of two animals. These animals, after being on the diet for 20 days, had little, if any, tetany, in spite of the fact that their serum calcium concentrations were 4.7 and 6.0 mg. per cent, respectively. They, however, showed fairly extensive calcification of their organs and it is possible that the mobilized calcium

<sup>2</sup> Minimal for growth and maintenance.

was deposited in the soft tissues instead of circulating in the blood stream. Similar low values for serum calcium without tetany were observed in unoperated animals fed optimal calcium-high phosphorus diets to which large doses of viosterol were added. These also had most extensive calcification of their blood vessels and kidneys.

A large animal of this group was bled by cardiac puncture on the 2nd day after parathyroidectomy and maintained on the low calcium diet. The serum calcium was 9.3 per cent and tetany had not yet appeared. On the 8th day the serum calcium fell to 5.1 mg. per cent and the animal exhibited severe tetany. The rat was then placed on the minimal calcium and phosphorus diet with 500 per cent viosterol and the tetany disappeared quite readily. It was bled again after 7, 17, and 75 days, and the serum calcium concentrations were 9.4, 10.0, and 9.1 mg. per cent, respectively.

*Effect of Viosterol and Stock Diet*—The stock diet differs from the preceding diet in that it is somewhat higher in calcium but slightly lower in phosphorus. Parathyroidectomized animals kept on this diet without viosterol varied with respect to their tetanic symptoms and the serum calcium concentration. The effect of the diet, however, was towards the alleviation of tetany and the development of normal or low normal serum calcium values. With the addition of viosterol to this diet the symptomatic improvement and the elevation of serum calcium concentration occurred much earlier and with increased regularity. The serum calcium levels are shown in Fig. 1.

*Effect of Viosterol and Low Calcium-High Phosphorus Diets*—This diet is faulty in two respects. It is extremely low in calcium (0.012 gm. per cent) and exceedingly high in phosphorus (1.780 gm. per cent). Even in animals with intact parathyroids it is frequently possible to induce latent or active tetany with such a diet. Parathyroidectomized animals fare very poorly on this type of ration and they may die or continue to have severe tetany, accompanied by very low serum calcium concentration. A few animals were kept on this diet with 1000 per cent viosterol for 60 days. The tetany in these animals gradually disappeared and their serum calcium concentration in the pooled blood was 9.2 as compared to 3.6 mg. per cent for the control group receiving no viosterol. The inorganic phosphorus, however, still remained high

(12.3 and 9.8 mg. per cent). At autopsy these animals showed fairly advanced calcification of blood vessels and kidneys.

*Effect of Viosterol and High Phosphorus and Minimal<sup>2</sup> Calcium Diet*—In spite of the addition of 1.0 gm. of  $\text{CaCO}_3$  to the preceding diet, it is still proportionally high in phosphorus. Parathyroidectomized rats do very poorly on this diet and remain in a state of tetany and hypocalcemia. Two rats fed this diet with the addition of 1000 per cent viosterol for 60 days had a serum calcium concentration of 10.0 mg. per cent. At autopsy most extensive calcification was found in the kidneys and in nearly all the blood vessels.

*Effect of Viosterol and the Steenbock Rachitogenic Diet*—In Paper I (10) it was shown that this high calcium-low phosphorus diet is very efficacious in relieving parathyroid tetany and in elevating the serum calcium to normal levels. The addition of viosterol to this diet caused no detectable improvement over the viosterol-free diet. The serum calcium concentrations of rats fed on this diet with 500 per cent viosterol for 20 days were 10.2 and 12.6 mg. per cent. With 1000 per cent viosterol the serum calcium levels were 12.7 at 60 days and 8.5 mg. per cent at 90 days. In the latter case there was moderate calcification of the small blood vessels.

*Effect of Viosterol and Calcium-Free and Calcium- and Phosphorus-Free Diets*—Although it was shown that viosterol may raise the serum calcium level of parathyroidectomized animals fed a diet containing only 0.012 gm. per cent of calcium, it was thought advisable to repeat the experiments with diets free of either calcium alone or of both calcium and phosphorus. The diet free of calcium alone was composed of 10 to 20 per cent purified casein, washed butter, and olive oil, and a phosphorus-containing salt mixture, so that the phosphorus content of the diet was approximately 0.500 gm. per cent. The remainder of the diet was made up with c.p. glucose. The calcium- and phosphorus-free diet contained similar amounts of glucose, fat, a salt mixture free of both calcium and phosphorus, and protein in the form of purified egg albumin. 2 per cent of a specially prepared oily solution of viosterol, having a potency 10,000 times that of cod liver oil, was added to the diets, so that they contained 20,000 per cent of viosterol in terms of cod liver oil.

After severe tetany was established in the operated animals by means of a low calcium diet, they were fed the experimental diets minus the viosterol for a few days in order to eliminate the possi-

TABLE I

*Effect of Viosterol on Serum Calcium and Inorganic Phosphorus of Parathyroidectomized Rats Fed Calcium- and Phosphorus-Free Diets and Calcium-Free Diets*

Rat No.	Days on viosterol	Serum Ca	Serum inorganic P	Remarks
Calcium- and phosphorus-free diets + 20,000 per cent viosterol				
		mg. per cent	mg. per cent	
1446	5	16.3	14.6	Calcification of heart muscle
1449	5	15.1	12.1	" " " "
1447	6	20.7		Extensive calcification of heart
1448	6	22.5		" " " "
1450	12	14.4	8.9	No calcification of soft tissues
1451	12	19.2	9.2	Extensive calcification of heart, kidneys, and aorta
1452	13	15.9	15.4	Moderate calcification of heart and kidneys
1453	13	16.2	15.0	Moderate calcification of heart and kidneys
1454	11	18.3	14.0	Moderate calcification of heart and kidneys
1455	11			Died. Most extensive calcification of heart and kidneys
1456	12	15.2	14.8	Most extensive calcification of heart and kidneys
1457	12	12.7	15.2	Calcification in cortico-medullary zones of kidneys
1460	6	15.2	14.2	Most extensive calcification of heart and kidneys. Moderate calcification of ascending aorta
1462	12	15.0	15.8	Most extensive calcification of heart and kidneys. Moderate calcification of ascending aorta
1463	12	18.0	15.0	Spotted calcification of heart and kidneys
1464	6	15.7	13.2	No calcification
1465	7	16.5	12.5	Slight calcification of ascending aorta and kidneys. Uretral calculi
1466	7	16.0	13.8	Slight calcification of ascending aorta and kidneys. Uretral calculi

TABLE I—*Concluded*

Rat No.	Days on viosterol	Serum Ca	Serum inorganic P	Remarks
Calcium-free diets + 20,000 per cent viosterol. P = 0.500 gm. per cent				
1435	11	12.8	7.8	Serum calcium before viosterol, 5.8 mg. per cent. No calcification
1437	11	14.3	8.7	Serum calcium before viosterol, 4.3 mg. per cent. No calcification
1436	17	14.4	8.2	Serum calcium before viosterol, 6.0 mg. per cent. Slight calcification of kidneys
1438	17	15.2	7.9	Slight calcification of kidneys
1439	17	16.0	7.5	" " " "
1440	10	14.9	6.1	No calcification of soft tissues
1441	4	13.6	7.2	" " " " "
1443	4	13.6	7.2	" " " " "
1444	5			Died. Calcification of heart muscles and aorta
1442	18	15.4	9.1	Moderate calcification of ascending aorta

bility of reabsorption of residual fecal calcium. The diets to which viosterol was added were then fed to the animals for varying periods until killed. At autopsy the thyroids were inspected carefully for the presence or absence of parathyroid tissue. The blood serum was analyzed for calcium and inorganic phosphorus. In order to obviate the possibility that through anorexia the amounts of viosterol ingested might have been inadequate, a few drops of viosterol 100 D, in addition to that contained in the diet, were fed to each animal separately for the first few days. A rough estimate of the food intake, the amount of feces, and the weights of the animals indicated that the viosterol-containing diets were consumed better than the low calcium diets without the viosterol. Most of the animals developed loose stools shortly after the viosterol administration, and the symptoms of tetany disappeared about the same time. The effects on the serum calcium and inorganic phosphorus concentrations are given in Table I. It is seen that large doses of viosterol are capable of raising the serum calcium of parathyroidectomized rats from tetanic to hypercalcemic levels even when no calcium was ingested. The levels of serum calcium and inorganic phosphorus were higher in the group



receiving the calcium- and phosphorus-free diet than in those receiving phosphorus but no calcium. This difference may be due either to the better consumption of the phosphorus-free diet and hence of viosterol, or to the abstraction of calcium by the ingested phosphorus and its excretion in the bowel as the insoluble phosphate salt. A similar difference in the serum calcium concentration was observed in unoperated animals on the respective diets (11).

Three of the rats had been bled by cardiac puncture shortly after operation, at the time when they exhibited severe tetany. Their serum calcium concentrations were 4.3, 5.8, and 6.0 mg. per cent, respectively. They were then placed on the calcium-free diet with 20,000 per cent of viosterol. Two were kept on this diet for 11 days and one for 17 days. Their serum calcium concentration in 11 days rose to 12.8 and 14.3 mg. per cent and to 14.4 mg. per cent in 17 days (Table I).

Autopsies on these animals revealed that both parathyroids had been completely removed, and that in some of the animals there was calcification in the myocardium, kidneys, and blood vessels (Table I).

#### DISCUSSION

From the results just enumerated it is seen that viosterol is capable of raising the serum calcium in parathyroidectomized rats, not only from tetanic to normal values, but also to hypercalcemic levels. This would seem to indicate that the activity of viosterol may be independent of the parathyroid glands.

The effects of viosterol were not only evident from the rise in the levels of serum calcium, but also from the symptomatic improvement and from the gain in weight, as shown in Fig. 2. It is interesting to note that the effects of small or moderate doses of viosterol did not manifest themselves for at least 10 days after its administration, and this delay may explain the negative results obtained by Greenwald and Gross, whose viosterol experiments lasted only 4 to 5 days—a period too short to expect an effect from small doses of viosterol in animals handicapped by thyroparathyroidectomy and by a calcium deficiency in the diet.

The experiments with the calcium-free diets are of special interest. Although it was shown that viosterol is capable of raising

the serum calcium levels of parathyroidectomized rats when their diets contained only 0.012 gm. per cent of calcium, the objection may be raised that the rise in serum calcium was due to an increased absorption and retention of the small amount of calcium ingested. In the experiments with the calcium-free diets the rise in the serum calcium to hypercalcemic levels in the absence of the parathyroids can only be ascribed to the activity of viosterol.

Recently Hess, Weinstock, and Rivkin (12) reported that they also were able to raise the serum calcium levels of parathyroidectomized dogs and monkeys, if the doses which they previously had found inactive were increased many times. Taylor, however, still maintains that there is "a close relationship between the overdosage of irradiated ergosterol and parathyroid function," and that "this relationship is . . . most probably, a direct one, namely, the stimulation of the parathyroid tissue by the sterol" (13) and, therefore, "the presence of intact parathyroid tissue is requisite for the production of hypercalcemia by viosterol" (14). The first assumption is drawn from certain similarities between the effects of overdosage of viosterol and parathormone. The second conclusion was based on the observation that viosterol was active, within 1 hour after its administration, in relieving parathyroid tetany in dogs subjected to ordinary thyroparathyroidectomy, but inactive in those in which all the aberrant neck tissues had been also excised,—an operative procedure which was thought to remove all possibility of parathyroid rests.

While it is quite possible that viosterol in therapeutic doses may act through or be regulated by the parathyroids, the experimental results of Taylor and his associates hardly prove or disprove it. In the first place, the presence or absence of parathyroid rests as an explanation for variations in the production of tetany is accorded too much importance, especially when the results can be explained by inadequate control of dietary factors. Even if it were true that ordinary thyroparathyroidectomy does not remove all parathyroid tissue, it is inconceivable that such small remnants of tissue should assume the function of four or more parathyroids within 1 hour after the oral administration of viosterol. Clinical experiences do not bear out such phenomenal compensatory activity on the part of the parathyroids. On the contrary, parathyroid tetany is a very frequent manifestation following the

removal of even one parathyroid, either accidentally in the course of thyroidectomy, or purposely, for the cure of *ostitis fibrosa cystica*. The occurrence of tetany is the more surprising in the latter case, since the parathyroids have been hyperfunctioning before the operative procedure and hence the remaining three or more parathyroids should be able to ward off the tetany. Secondly, in the experiments of Taylor the response to the oral administration of viosterol was very prompt, according to him, through stimulation of parathyroid rests to greater activity; yet, when a similar dose of viosterol was given intravenously to a dog with intact parathyroids, the viosterol effects in raising the serum calcium became evident only after 24 to 48 hours. Obviously the prompt response in relieving the tetany by oral administration can hardly be attributed to the viosterol. Thirdly, no serum calcium determinations were made in the operated group and, even if the levels of serum calcium were increased by viosterol, it does not necessarily follow that parathyroid tissue was called to functional activity, for it is possible that large doses of viosterol may have acted upon the organic matrix of the bones (10, 15) and thereby released calcium for the amelioration of the tetanic symptoms.

The bulk of evidence brought forward by Taylor to demonstrate the similarity between viosterol and parathormone may be applicable to large doses, and not to small amounts of these substances. While the effects of therapeutic doses of viosterol are fairly well established, little is known of the effects of small doses of parathormone in facilitating calcium retention and deposition in the rachitic organism. Although hyperplasia of the parathyroids has been described in rickets and osteomalacia, it is not entirely certain whether it is an expression of hyper- or hypofunction of these glands. The experiments of Bischoff (16) would seem to indicate that the parathyroid hyperplasia in rickets constitutes hypofunction, since he was able to cause retention of calcium in rachitic dogs by the injection of parathyroid extract. On the other hand, the experiments of Pappenheimer (17) and of Waltner (18) suggest the opposite view, namely hyperfunction. The former has shown that the removal of the parathyroids in rats increased their resistance to rickets, and the latter has observed that the injection of parathormone into rats intensified their

susceptibility to this malady. Preliminary experiments performed in this laboratory seem to support the observations of Pappenheimer and of Waltner. When parathormone was injected into rachitic rats, the rachitic process was intensified and the shafts of the bones showed areas of decalcification instead of healing and improved calcification, as would be expected were parathormone and viosterol identical in their activity.

The changes in the serum calcium and inorganic phosphorus of normal or parathyroprivic animals induced by parathormone and viosterol are also quite different. Whereas viosterol raises the levels of both calcium and phosphorus in the blood, parathormone increases the calcium concentration but lowers the inorganic phosphorus.

With the experimental data at hand, no definite conclusions can be drawn as to the relationship of viosterol and the parathyroids. It is evident, however, that large doses of viosterol may increase the serum calcium level in the absence of the parathyroids and of calcium in the diet. The effect is the opposite to that observed when small doses are administered. Therapeutic doses of viosterol raise the serum calcium and inorganic phosphorus levels in the blood and the stream of flow is toward the osseous tissue, while large doses cause dissolution of the osseous matrix and a flow of lime salts to the blood,—to be excreted in the urine and feces or to be deposited in the soft tissues.

#### SUMMARY AND CONCLUSIONS

1. Viosterol in fairly large doses is capable of raising the serum calcium levels and of ameliorating the symptoms of tetany of parathyroidectomized rats kept on very low calcium diets.

2. Large doses of viosterol may raise the serum calcium of parathyroidectomized rats from tetanic to hypercalcemic levels, even in the absence of either calcium alone or of both calcium and phosphorus from the food.

3. These experiments would seem to indicate the activity of viosterol in raising the serum calcium levels may be independent of the parathyroids.

4. The relation of the parathyroids to the activity of therapeutic doses of viosterol is discussed.

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## CALCIUM AND PHOSPHORUS STUDIES

### III. THE SOURCE OF EXCESS SERUM CALCIUM IN VIOSTEROL HYPERCALCEMIA\*

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All experimental and clinical observations make it evident that the administration of small or moderate amounts of viosterol to human beings or animals facilitates calcium retention and improves ossification. Large doses, however, cause an increase of calcium in the blood, which is either excreted or deposited in the soft tissues of the body. That the bones are the source of calcium for the viosterol hypercalcemia may be deduced from the experiments of Brown and Shohl (1), and of Light, Miller, and Frey (2), who have demonstrated that large doses of viosterol cause a negative calcium balance and a diminution of the ash content of the bones. These findings have been partially corroborated by Watchorn (3). Hess, Weinstock, and Rivkin (4) and Shelling (5) produced viosterol hypercalcemia in animals on diets very low in calcium; and György (6) and Shelling (5) have described areas of decalcification in the upper part of the tibiae of young rats fed on such diets.

Although the experiments just cited indicate plainly that the source of calcium in viosterol hypercalcemia is the osseous tissue, Jones, Rapoport, and Hodes (7) conclude, from their own experiments, "that the source of the excess calcium in irradiated ergosterol hypercalcemia is the food and not the body tissue." They criticize the experiments of Hess and his coworkers on the ground

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that the diets used (the composition of which is not given) may have contained enough calcium to cause the hypercalcemia. They might have directed similar criticism against the results of Watchorn (8), since the diet used by her was not calcium-free but contained at least 15 to 50 mg. per cent of calcium, which was entirely neglected in the intake figures.

While the experimental results of Jones and coworkers seem entirely valid, yet the conclusions which they draw from them are not warranted. In the first place the amounts of viosterol used by them were only moderately high (100 to 200 times overdosage). If larger doses had been used, the results might have been entirely different. Secondly, the calcium-free diet (lard and glucose) was also free of protein and electrolytes<sup>1</sup> and it is quite possible that a diet so grossly unbalanced and defective played some part in preventing the excess solution of calcium and phosphorus in the blood, especially since the solubility product constant of calcium and phosphorus in the blood must be influenced by, and therefore must depend, to a large extent, on the presence of adequate amounts of protein and electrolytes as demonstrated by Holt, La Mer, and Chown (9), Shelling, Kramer, and Orent (10), and Shipley and Holt (11). Thirdly, the determination of the serum calcium measures only the concentration, but gives no indication as to the direction of flow. Since the excreta were not analyzed, it is conceivable that the excess calcium derived from the tissues was rapidly excreted in the urine and feces.

In Paper II (12) it was shown that the serum calcium of parathyroidectomized rats can be raised, by viosterol, from tetanic to hypercalcemic levels, even when the diets were lacking either in calcium alone or in both calcium and phosphorus. If viosterol

<sup>1</sup> After this paper was written, Jones and Rapoport (Jones, J. H., and Rapoport, M., *J. Biol. Chem.*, **93**, 153 (1931)) reported that, contrary to their previous inability to raise the serum calcium to marked hypercalcemic levels in dogs fed calcium-free diets, they were able to produce hypercalcemia with larger doses of viosterol and with a change in diet. The last diet used was composed of 99 parts of yellow corn and 1 part of NaCl. In contrast to their previous diet, which was free of protein and electrolytes as well as of calcium and of phosphorus, the corn diet contained protein, about 40 mg. per cent of calcium, a small amount of phosphorus, and a moderate quantity of other electrolytes. Even this diet was higher in calcium than the diet previously used by Shelling (5).

is capable of producing hypercalcemia in the absence of parathyroids; its activity should be more manifest in the normal animal. The following experiments were carried out to ascertain: (1) the effects of large doses of viosterol on the levels of serum calcium of rats fed diets free of either calcium or of both calcium and phosphorus, and (2) the effects of such a régime on the excretion of calcium and phosphorus.

#### EXPERIMENTAL

Young rats ranging in weight from about 50 to 120 gm. were used. They were kept on the stock diet from weaning until the beginning of the experiment. The experimental diets were fed for a few days prior to the viosterol feeding in order to eliminate the possibility of reabsorption of residual fecal calcium and phosphorus. The viosterol-containing diets were then given for varying periods until the animals began to show signs of ill health, as evidenced by a rapid decline in weight, lessened activity, and loose stools. They were then killed and autopsied. The blood serum was analyzed for calcium and inorganic phosphorus.

The diets were of two types: (1) calcium-free and (2) calcium- and phosphorus-free. Both contained 20 per cent protein, 1 per cent cod liver oil, 5 per cent olive oil, C.P. glucose, and 2 cc. of viosterol 10,000 D. The calcium-free diet had purified casein and a phosphorus-containing salt mixture, so that the phosphorus content was about 0.500 gm. per cent. The calcium- and phosphorus-free diet contained purified egg albumin and a salt mixture free of both calcium and phosphorus. Vitamin B was not given. The omission of vitamin B from the dietary for short experimental periods was thought more desirable than the addition of mineral contaminants from yeast or wheat germ.

#### Results

*Effects of Viosterol and Calcium-Free Diets*—Thirty-three rats were used in this group. Since many of the animals were small, the blood of three or four had to be pooled to obtain enough serum for calcium analysis. Hence only nine determinations of serum calcium were made. The serum calcium concentration varied from normal to moderate hypercalcemic levels (9.5 to 15.6 mg. per cent). Most of the animals developed diarrhea and lost



weight soon after the viosterol feeding. At autopsy all showed evidence of emaciation and osteoporosis. The intestines were distended with gas and slimy yellowish material. Some of the animals showed calcification in the kidneys or blood vessels, while others were free of metastatic calcification. The toxicity of viosterol did not express itself in calcification of the soft tissues

TABLE I

*Effect of Viosterol (20,000 Per Cent) on Serum Calcium and Inorganic Phosphorus of Rats Fed Calcium-Free Diets (P = 0.500 Gm. Per Cent)*

Rat Nos.	Average weight	Days on viosterol	Serum Ca	Serum inorganic P	Remarks
	gm.		mg. per cent	mg. per cent	
2822-3	100	8	15.6		Calcification of blood vessels and kidneys
2824	100	8			Died; calcification pronounced
2825-7	100	11	13.8	9.9	Marked calcification of aorta
2782	110	13	10.7	9.5	Moderate calcification of blood vessels
2784-7	110	13	10.7	9.5	Moderate calcification of blood vessels
2783	110	12			Died; moderate calcification of blood vessels
2796-7	90	23	13.6	8.5	Slight calcification of aorta
2798-9	90	23	14.4	8.8	" " " "
2800-2	90	17	15.0	8.8	No metastatic calcification
2890-4	45	12	9.5		" " " Bones
					markedly osteoporotic
2895-8	45	10	12.4		Calcification in myocardium of 1 rat; osteoporosis
2899	45	19	11.0		No gross abnormal calcification; marked osteoporosis
2900-3	45	19	11.0		No gross abnormal calcification; marked osteoporosis

alone but in a rapid dehydration, wasting, and tissue necrosis. The results are given in Table I.

*Effect of Viosterol and Calcium- and Phosphorus-Free Diet*—This diet was fed to 55 rats. Eighteen analyses of pooled blood for serum calcium and thirteen for inorganic phosphorus were made. The concentrations are shown in Table II. The serum calcium levels varied

between 12.9 and 19.7 mg. per cent. The concentrations of serum calcium were somewhat higher, and the frequency and extent of

TABLE II

*Effect of Vioosterol (80,000 Per Cent) on Serum Calcium and Inorganic Phosphorus of Rats Fed Diets Free of Both Calcium and Phosphorus*

Rat Nos.	Average weight	Days on vioosterol	Serum Ca	Serum inorganic P	Remarks
			mg. per cent	mg. per cent	
2722-4	65	16	15.5	9.3	No calcification in soft tissues
2732-5	55	12	13.8	7.5	" " " " "
2729-31	65	10	13.3	8.5	Slight decalcification of trabeculae of tibiae
2725-8	60	19	12.9	8.9	Slight calcification in soft tissues
2765-7	49	22	14.9	9.8	Slight calcification in soft tissues
2769-71	52	25	14.5	8.0	Beginning calcification of kidneys; osteoporosis
2821-5	48	16	12.9		Moderate calcification of blood vessels
2623-4	55	12	18.9	8.6	Calcification of coronaries and myocardium in 4 rats; moderate calcification of kidneys in 5; urethral calculi in 2; dental caries in 2; decalcified incisors in 2; bones osteoporotic in all
2625-6	55	12	19.2		
2627-9	55	12	19.5		
2628-30	80	12	19.0	8.9	Calcification in myocardium and kidneys
2831-3	80	18	15.1	8.1	Calcification also in aorta
2834-6	80	11	15.5	8.3	Metastatic calcification in 1 rat
2837-9	85	18	13.8	7.9	Moderate calcification of heart and kidneys
2904-6	45	7	19.7		Spotted calcification of myocardium
2907-8	45	5	13.5	15.0	Spotted calcification of myocardium. Bones very soft
2909-10	45	7	16.7		No calcification in soft tissues
2911-14	42	14	15.1	9.0	Moderate calcification of blood vessels

metastatic calcification were slightly greater in this group than in the preceding group. The possible explanation for these differences will be discussed later.

*Effect of Viosterol on Excretion of Calcium and Phosphorus*

Further proof that the tissues may be the source of excess calcium in viosterol overdosage was obtained from calcium and phosphorus determinations on the urine and feces of rats fed the calcium- and phosphorus-free diet with 20,000 per cent of viosterol. The animals were kept in a Hopkins-Gamble metabolism cage and fed the experimental diet without viosterol for a few days in order

TABLE III

*Effect of Viosterol on Excretion of Calcium and of Phosphorus*

In Experiment 1, a calcium- and phosphorus-free diet plus viosterol was fed.

In Experiment 2-a a calcium- and phosphorus-free diet without viosterol was fed; in Experiment 2-b viosterol was added to the same diet.

In Experiment 3-a the diet contained phosphorus but no calcium and no viosterol; in Experiment 3-b viosterol was added.

	Ca excretion		P excretion		Excretion per rat per day	
	Urine	Feces	Urine	Feces	Ca	P
	mg.	mg.	mg.	mg.	mg.	mg.
Experiment 1	275.0 (75.0%*)	90.0 (25.0%)	170.0 (75.5%)	55.3 (24.5%)	30.0	18.7
“ 2-a	3.0 (5.0%)	55.0 (95.0%)	45.0 (64.3%)	25.0 (33.7%)	3.6	4.4
“ 2-b	1050.0 (97.0%)	38.0 (3.0%)	1550.0 (98.0%)	25.0 (2.0%)	17.0	24.6
“ 3-a	2.0 (4.8%)	40.0 (95.2%)	45.0 (17.0%)	220.0 (83.0%)	10.5	66.7
“ 3-b	22.5 (5.2%)	408.0 (94.8%)	100.0 (10.0%)	904.0 (90.0%)		

\* Per cent indicates fraction of total excretion.

to eliminate the possibility of reabsorption of residual fecal calcium and phosphorus. In the first experiment four young litter mates, weighing about 55 gm. each, were used. The urine and feces of the viosterol-free period were discarded and those of the viosterol period of 3 days were analyzed for calcium and phosphorus. The results are given in Table III (Experiment 1). It is seen that, in spite of the entire absence of calcium and of phosphorus from the diet, the excretion of calcium was 30.5 mg. and that of phosphorus 18.7 mg. per rat per day. The animals were

killed because they developed loose stools and appeared sick. Their serum calcium concentration was 12.9 mg. per cent. One rat had multiple plaques of calcification in the heart muscles, and the bones of all four were soft and showed areas of decalcification.

In the second experiment four larger rats were used, since it had been previously observed that such animals survived the effects of viosterol overdosage much longer than did very young ones. They were given the calcium- and phosphorus-free diet without viosterol for two successive periods of 2 and 4 days each and then the same diet with 20,000 per cent of viosterol for a third period of 16 days. The urine and feces of the first viosterol-free period were discarded, while those of the succeeding two periods were analyzed for calcium and phosphorus. The results are given in Table III (Experiment 2). It is seen that in the viosterol-free or second period the excretion of calcium was 3.6 mg. and that of phosphorus 4.35 mg. per rat per day; whereas, during the viosterol period the daily calcium excretion was 17.0 mg. and that of phosphorus 24.6 mg. per rat per day. The excess excretion of phosphorus over calcium may be due to the destruction of soft tissue over a period of 16 days, as was evidenced by emaciation and loss of weight.

In the third experiment two rats, weighing 158 and 175 gm. respectively, were used. They were fed the calcium- and phosphorus-free diet for 2 days, and the urine and feces were discarded. On the 3rd and 4th days they were fed the calcium-free diet to which 5.0 gm. per cent of anhydrous  $\text{Na}_2\text{HPO}_4$  were added, so that the diet contained 1.090 gm. per cent of phosphorus, but no calcium. On the 5th day and for 7 days thereafter they were fed the same diet to which 20,000 per cent of viosterol had been added. The excreta were analyzed for calcium and phosphorus. The results are given in Table III (Experiment 3). It is seen that the addition of excess phosphorus to the calcium- and viosterol-free diet resulted in the excretion by the bowel of 10.0 mg. of calcium per rat per day and that the viosterol caused an added excretion of this element through the same channel.

#### DISCUSSION

From the results of the above experiments it is evident that the excess serum calcium in viosterol hypercalcemia may be derived from the tissues. This is shown: (1) by the occurrence of hyper-

calcemia in rats fed calcium- and phosphorus-free diets to which large doses of viosterol were added; (2) by the excess excretion of calcium on such dietaries; and (3) by the presence of Roentgenological and histological evidence of decalcification of the bones.

It is interesting to note that the serum calcium levels were slightly higher in the rats not receiving calcium or phosphorus in their diets than in those receiving phosphorus but no calcium. Similarly, the incidence and extent of metastatic calcification were somewhat greater in the former group than in the latter. The difference may be explained as follows: From the metabolism experiment described in this paper it is seen that in the absence of both calcium and phosphorus from the diet the increased excretion of both calcium and phosphorus caused by viosterol occurs in the urine. When, however, phosphorus is added to the diet, the excretion of calcium is shifted from the urine to the feces. Apparently, in the absence of both calcium and phosphorus from the diet, the excess calcium and phosphorus in the blood resulting from the abstraction of these elements from the bones by viosterol is excreted through the urine. But when the blood phosphorus is increased through the ingestion of dietary phosphorus to such an extent that the kidneys are unable to eliminate this excess and that derived from the bones, a second path of excretion is opened, namely, the bowel. The excretion of phosphorus into the bowel as the insoluble calcium salt leaves the concentration of calcium in the blood at a comparatively lower level. Thus in the groups not receiving phosphorus the calcium in the blood, passing from the bones to the channel of excretion, may be greater than in those receiving it. Similarly, as in the experiments of Jones *et al.*, if the diet be deficient in protein and electrolytes, the resultant paucity of the blood, especially of the latter, decreases the solubility product constant of  $\text{Ca}^{++}$  and  $\text{PO}_4^{--}$  and less of these ions are possible in solution in the blood. Hence the possible absence of marked hypercalcemia.<sup>1</sup>

The results of the metabolism experiments reported here also demonstrate the importance of the ratios of calcium and phosphorus of the diet in determining the paths of excretion of these elements,—a fact frequently stressed by Shohl<sup>2</sup> and Bennett (13).

<sup>1</sup> Dr. Shohl led the discussion of a phase of the subject at the symposium on calcium and phosphorus metabolism conducted at the meeting of the

Brown and Shohl (4) have shown that the addition of increasing amounts of ~~viosterol~~ to the Sherman stock diet caused a shift of the excretion of both calcium and phosphorus from the feces to the urine. Watchorn (3), however, concluded from her experiments, that, as a result of ~~viosterol~~ administration "the urinary calcium is greatly increased, but not the phosphorus." It is obvious, from Watchorn's intake figures, that the diet used by her contained twice as much phosphorus as calcium and hence the excess phosphorus was excreted through the intestine rather than through the kidneys.

The present metabolism experiments also throw some light on the problem of absorption of calcium and phosphorus from the intestines. The question as to whether rickets is to be attributed to a diminished absorption of lime salts by the small intestines or to an increased excretion of these elements in the large intestines after absorption has taken place is as yet not definitely settled. The metabolism experiments of Schabad (14), Schloss (15), Grosser (16), and Telfer (17) indicate that during the active stage of rickets there is a loss of calcium and phosphorus in the feces and that during the healing periods the excretion of these elements in the feces is diminished, but the calcium excretion in the urine is increased. This alteration in the path of calcium excretion during antirachitic treatment led the above investigators to believe that rickets is associated with a diminished capacity of the intestine to absorb calcium and phosphorus and that with improvement in absorption healing takes place. The experimental results of Orr, Helt, Wilkins, and Boone (18) on the effect of ultra-violet radiation on the healing of rickets are in agreement with the data of the above experiments. These investigators, however, are cautious in drawing definite conclusions as to the mode of action of ultra-violet radiations in the healing of rickets. While they are inclined to believe that the increased retention of lime salts during healing is due to better absorption of calcium and phosphorus from the intestines, they also consider a decreased excretion as a factor in retention. Direct proof that the faulty metabolism in rickets may be due to an increased excretion of lime salts rather than to

a diminished absorption is offered by the experiments of Bergeim (19) in which the calcium and phosphorus of the contents of different portions of the intestines of rachitic rats were determined while absorption and excretion were in progress. The results of the present metabolism experiments with calcium-free diets, and especially with the diet to which phosphorus was added, seem to support the view of Bergeim. They indicate that large amounts of lime salts may be excreted from the blood into the intestine, and that their excretion into the intestine is governed by factors which limit the solubility of calcium and phosphorus in the blood. Thus, it is conceivable that in rickets and osteoporosis calcium and phosphorus are absorbed in normal amounts in the upper intestinal tract and, depending upon the solubilities and the ratios of these ions in the blood, their excess is excreted in the colon as the insoluble salt.

With the advent of viosterol as an antirachitic agent the question of gut absorption became even more confusing, since small doses of viosterol promote calcium retention, while large doses may cause a negative calcium balance and demineralization of the bones as shown by Brown and Shohl (1), Light, Miller, and Frey (2), and Watchorn (3). The most accurate studies on the effect of viosterol on calcium and phosphorus metabolism are those of Brown and Shohl, since their data were based on experiments in which the calcium-phosphorus ratios of the diets were adequately controlled. Their figures indicate that on the Sherman stock Diet B and also on the Steenbock rachitogenic diet, small or moderate doses of viosterol caused an increased retention of both calcium and phosphorus and that large doses induced a negative balance and a demineralization of the skeleton. With increasing amounts of viosterol, there was also a shift in the paths of excretion of lime salts from the feces to the urine. They do not attach special significance to the change in the path of excretion produced by viosterol nor do they attempt to attribute viosterol activity to better absorption of calcium and phosphorus from the intestines, since they are aware of the difficulty of differentiating the calcium and phosphorus which escaped absorption from that which was absorbed and then excreted. Harris and Innes (20), however, made use of the data of Watchorn (3) and of Brown and Shohl (1) in order to calculate the percentage of gut absorption.

They subtract the fecal calcium and phosphorus from the total intake and the remainder, including urinary calcium, expressed in percentage of intake, they consider as the amount absorbed.  $\text{Ca intake} - \text{fecal Ca} = \text{absorbed (i.e. urinary + retained Ca)}$ .

$$\text{Per cent Ca absorption} = \frac{\text{retained} + \text{urinary Ca}}{\text{Ca intake}}$$

They conclude that with small or moderate doses of viosterol there is an increase and with large doses a decrease in gut absorption of lime salts. However, in considering the results of Brown and Shohl and of those reported in this paper such deductions are not entirely justifiable, since fecal calcium excretion may vary not only with the degree of absorption but also with the physicochemical state of  $\text{Ca}^{++}$  and  $\text{PO}_4^{--}$  in the circulating fluids, irrespective of absorption. The fallacy of determining gut absorption by subtraction of fecal excretions from intake calcium is even more striking when the results of the metabolism experiments with calcium-free diets are considered. Thus, if the calculations of Harris and Innes are applicable, the large excretion of calcium through the kidneys in the first and second experiments would indicate that the rats had ingested 0.275 and 1.050 gm. of calcium, respectively, and the absorption had been nearly 100 per cent in each instance, whereas in reality none was ever ingested at all. Similarly, in the succeeding experiment in which the path of calcium excretion was changed to the feces by the addition of  $\text{Na}_2\text{HPO}_4$  to the diet, the large excretion of calcium in the feces would mean that 0.408 gm. of calcium had been added to the diet and nearly 100 per cent of it had remained unabsorbed, whereas in reality none had been given. Obviously, such calculations do not determine the degree of absorption or non-absorption.

That the therapeutic effects of viosterol are not necessarily due to an increased gut absorption is also evident from the experiments of Bauer *et al.* (21). In a preliminary note, Bauer and Marble (22), laying stress on the alteration of the path of excretion of calcium as an indication of the degree of absorption from the gut and also on the changes in fecal pH after antirachitic therapy, evolved a theory that viosterol acts by altering the composition of the pancreatic juice towards the acid side and thereby facilitates calcium absorption. However, in a later communication



(21), in which they report studies on the effect of viosterol on the free HCl of the gastric juice and on the acid-base composition of the pancreatic juice, they disprove their original hypothesis. Contrary to their expectations, viosterol produced a reduction in the free HCl and an increase in the alkalinity of the pancreatic juice instead of an increase in acidity.

Modern metabolic and physicochemical studies (9, 10, 23) make it apparent that conditions in the tissues and body fluids which are influenced by the calcium and phosphorus intake determine, to a large degree, the paths of excretion and the ultimate fate of calcium and phosphorus which remain in the body. One of these conditions is the limitation of the amount of lime salts possible in solution in the body fluids, or the solubility product constant ( $K_{s.p.}$ ) of  $\text{Ca}^{++}$  and  $\text{PO}_4^{--}$ . An excess of the solubility of these ions in the blood is either deposited in the tissues or is excreted. The choice of retention or excretion rests with the needs of the body; i.e., with the receptivity of the calcifying tissues. In the growing organism, on an optimal calcium, phosphorus, and vitamin intake, the equilibrium at the site of deposition is toward the positive side and the slight excess of  $K_{s.p.}$  of  $\text{Ca}^{++}$  and  $\text{PO}_4^{--}$  in the blood, occasioned by ingestion, is deposited in the growing osseous tissue. The path of excretion of the unused calcium and phosphorus is determined by the ratio of these ions. On a high calcium-low phosphorus diet the excess calcium is first excreted through the kidneys, and, when its concentration in the urine reaches a maximum, it is then excreted through the bowel as the insoluble phosphate salt, and thus robs the body also of phosphorus. In the growing organism, although the bones are receptive to lime salt deposition (10, 11, 24, 25), the constant loss of these elements by excretion prevents their reaching the bones in the proper concentrations for deposition, and rickets results. An increase in the ratio of phosphorus to calcium in the blood by the ingestion of an excess of phosphorus results in the excretion of the excess, first by the kidneys up to a maximum, and then by the bowel as the insoluble calcium salt. The abstraction of calcium by the excess phosphorus results in rickets, or more commonly, in osteoporosis.

In low phosphorus rickets the administration of antirachitic agents results in calcium and phosphorus retention and their

deposition in the bones. The excess of calcium over phosphorus necessary for bone formation is excreted by the kidneys; hence the absolute decrease in excretion of both elements in the feces and the increase of calcium in the urine. In the low calcium type of rickets, the opposite effect is noted; namely, an increased excretion of phosphorus in the urine. These effects, however, are only secondary to lime salt retention and do not explain the exact mechanism as to the activity of antirachitic agents.

With increasing doses of antirachitic substances, such as viosterol, resorption exceeds deposition and the excess of lime salts in the blood above their solubility product constant is excreted in the urine and feces,—the path of excretion depending on the increase of these ions and their ratios in the blood, and hence on the intake.

Viosterol in very large doses causes general toxic effect, since it may produce necrosis and inflammation without calcification. Histologically, the effect of excessive doses on the osseous tissues expresses itself not only by the removal of lime salts but also by actual dissolution of the organic matrix, so that deposition in the bones becomes an utter impossibility. In the presence of optimal amounts of both calcium and phosphorus in the diet the excessive calcium and phosphorus in the blood are derived both from the bones and from the food. The excess of the solubility of these ions in the blood is excreted by the urine and feces,—the increase being reflected more in the urine than in the feces, as shown by experiments of Brown and Shohl. The excess which escapes excretion is deposited in the soft tissues. In the absence of both calcium and phosphorus from the diet, the lime salts liberated by the dissolution of bone, as a result of excessive doses of viosterol, are excreted mainly by the kidneys, as demonstrated in the present metabolism experiments. The excess phosphorus in the blood, brought about by the ingestion and also by the liberation from the bones, is excreted by the kidneys and bowel. Since the kidneys are capable of excreting phosphorus only up to a limited concentration, the remainder must be excreted by the large intestine as the *insoluble* salt of calcium. Hence the shift of calcium excretion from the urine to the feces. An attempt on the part of the kidneys to excrete both calcium and phosphorus in marked concentration will usually result in precipitation of lime salts in its

substance or in the formation of calculi; and the excretion of phosphorus by the bowel in a form other than the insoluble will usually evoke a diarrhea.

#### SUMMARY AND CONCLUSIONS

1. Viosterol hypercalcemia may be induced in rats fed calcium-free diets.

2. The serum calcium levels are somewhat higher and the incidence of metastatic calcification is more frequent and widespread in rats fed large doses of viosterol and diets free of both calcium and phosphorus than in those fed similar rations but free of calcium alone.

3. With diets free of both calcium and phosphorus viosterol causes an increased excretion of both calcium and phosphorus in the urine, but when an excess of phosphorus is added to the diet the excretion of calcium shifts to the feces.

4. Rats kept on calcium-free diets to which large doses of viosterol are added show histological and Roentgenological evidence of bone decalcification.

5. Viosterol may cause toxic symptoms in animals without producing abnormal calcification in the soft tissues.

6. The physicochemical considerations of the mechanism of the excess excretion of calcium and phosphorus and of metastatic calcification are discussed and the fallacy of determining gut absorption of calcium from fecal and urinary excretions is also pointed out.

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## **DIFFUSIBLE AND NON-DIFFUSIBLE BLOOD SERUM CALCIUM FOLLOWING INTRAVENOUS INJECTIONS OF CALCIUM SALTS**

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The administration of calcium salts by various routes is a common practice in therapeutics. It is recognized that such administration is usually followed by some rise in blood serum calcium, depending on the dosage, method of administration, etc. Furthermore, some years ago, certain workers, Rona and Takahashi (1) and Cushny (2), clearly demonstrated that blood serum calcium could be divided into two fractions by a membrane impermeable to proteins. Since that time, considerable interest has been manifested in this phase of the subject. Various methods have been advanced for the estimation of the two fractions and such estimations have been reported by many authors both in normal serum and in a variety of pathological conditions.

Even of more interest is the fact that some attempt has been made to designate certain physiological functions to each fraction. It was natural to assume *a priori* that the diffusible portion, including the ionized calcium, would be more active physiologically. In this regard, brief comment may be made on the relation of the parathyroid hormone to diffusible and non-diffusible calcium, although the present work is not directly concerned with that subject. In a recent paper Gunther and Greenberg (3) have expressed the opinion that, in infantile and parathyroid tetany, and following the administration of parathyroid hormone, the changes in serum calcium are due almost entirely to a variation of the diffusible fraction, and they present evidence in favor of this view. Such a conclusion is entirely at variance with that of certain workers who assume that the cerebrospinal fluid calcium

content (which changes only slightly in the presence of parathyroid hormone variations) represents the diffusible calcium content of the blood. Greenberg (4), Morgulis and Perley (5), and Hertz (6) have demonstrated that such an assumption is quite untenable when blood serum calcium is varied from normal values. However, most workers in this field find changes in both fractions, although the diffusible calcium very often shows the greater change and often precedes more or less parallel changes in the non-diffusible calcium. An indication that the non-diffusible calcium may be of some physiological importance is contained in the work of Stewart and Percival (7) which suggests that this fraction is the one necessary for the coagulation of blood.

If we grant that there may be a difference in the physiological activity of the two fractions, it is of interest to follow the changes in each after calcium administration or, in other words, to determine whether one or both fractions are responsible for the increase in total blood serum calcium. Definite conclusions in this regard might possibly modify the therapeutic use of this substance.

Solutions of the calcium salts used in the following experiments (chloride, lactate, and gluconate) proved to be wholly diffusible by the method employed and so would naturally be expected to cause a rise in that fraction of the blood serum calcium. Changes in the other fraction might follow, depending on certain conditions. If the normal proportion existing between the two fractions is the result of a chemical equilibrium, some rise in the non-diffusible fraction would be expected to result from an addition of diffusible calcium salts. Evidence for such a supposition is found in the work of Loeb and Nichols (8) who dialyzed blood serum against various concentrations of calcium chloride. Their results show that the non-diffusible calcium of the serum is a function of the concentration of diffusible calcium at normal hydrogen ion and protein concentrations. Furthermore, there is a greater proportion of non-diffusible calcium in the presence of low calcium concentrations and they suggest that under such circumstances a greater proportion of the calcium is required for the formation of calcium-protein complexes. (This finding may explain the results of Gunther and Greenberg (3) who report practically normal amounts of non-diffusible calcium when the diffusible fraction is lowered in parathyroid tetany.) Loeb (9) by similar methods had

previously shown the combined or non-diffusible calcium to be a function of the protein and hydrogen ion concentrations. Mar-rack and Thacker (10), dialyzing serum protein solutions and also serum against calcium chloride solutions, reached the same conclusions in this regard as the above authors. If such an equilibrium does exist, a change in both fractions might be expected in parathyroid hormone variations irrespective of the fact that the primary variation may concern only the diffusible fraction; and such are the findings of several workers, as previously mentioned.

Also, a question of more direct concern in this work is that of serum protein saturation with calcium; *i.e.*, whether or not the serum protein is capable of combining with calcium above the normal amount in the presence of an increased diffusible calcium content. There are numerous evidences in the literature that the combined calcium concentration and hence, to some extent, the total calcium concentration of various body fluids (blood serum, cerebrospinal fluid, ascitic fluid, etc.) is a function of the protein concentration. However it is quite possible that the proteins of such fluids would be capable of combining with more calcium in the presence of greater calcium concentrations. The results of Loeb and Nichols (8), obtained by the use of artificial systems, answer this question in the affirmative. They believe serum protein saturation to be incomplete. Using their results, Greenberg and Gunther (11) have shown that the calcium combined with protein bears a mathematical relation to the calcium present and will increase to a certain limiting value in the presence of increasing concentrations of calcium chloride. Also, the fact that the administration of parathyroid hormone to the normal subject will cause some increase in non-diffusible calcium without any change in serum protein is additional evidence in this regard (Snell (12)). Finally Morgulis and Perley (5), although not interested in this phase of the subject, report certain experiments in which the diffusible calcium was determined by dialyzing blood serum against equal volumes of cerebrospinal fluid from the same animal, before and after intravenous injections of calcium chloride solution. Although the total serum calcium increased, there was no appreciable change in the percentage of diffusible calcium, indicating that the non-diffusible fraction must have increased proportionately with the diffusible fraction.



Consequently, from the literature, it must be concluded that there is an equilibrium between diffusible and non-diffusible calcium and that an increase in both fractions would be expected following the injection of diffusible calcium salt solutions. It is our purpose to test this experimentally, on dogs, by following the concentration of both forms of calcium after the intravenous injection of such solutions.

### *Methods*

Normal unanesthetized dogs were used as experimental animals in all cases. Calcium salts were administered intravenously, as the changes in blood serum calcium resulting from this method are most striking and consequently are most readily investigated. Blood samples for calcium analyses were taken by cardiac puncture before and at certain intervals following calcium administration, until the total serum calcium had returned to the normal level. Duplicate analyses for total serum calcium and also for ultrafiltration necessitated blood samples from 20 to 25 cc. in volume.

Total blood serum calcium determinations were made by the Clark-Collip (13) modification of the Kramer-Tisdall method. 2 hours were allowed for the precipitation of calcium oxalate.

Due to an apparent appreciable loss of calcium during ultrafiltration in certain of the earlier experiments, the calcium content of the collodion sacs after ultrafiltration was later determined as a routine procedure. The method used was as follows: Two sacs from duplicate experiments were placed in a large Pyrex test-tube (20 × 3 cm.). 5 cc. of perchloric acid were added. The contents of the tube were boiled over a micro burner, bumping being prevented by a glass bead. The sacs quickly decomposed and boiling was continued to dryness. The residue was dissolved in about 1 cc. of distilled water and the solution transferred quantitatively to a centrifuge tube by means of a capillary pipette, the total volume being 4 cc. After the addition of 1 cc. of 4 per cent ammonium oxalate solution, a drop of methyl red indicator solution was added. If necessary a drop of dilute hydrochloric acid was added just to produce an acid reaction to the indicator. The procedure was continued as in the determination of total serum calcium.

Serum protein determinations were made in a few experiments; the method of Greenberg (14) was used.

Hydrogen ion concentration determinations on the serum were not made as a routine procedure. However, in certain instances in which such determinations were made by the method of Hastings and Sendroy (15) the results were well within the limits shown by Greenberg and Gunther (11) to have no effect on the diffusible calcium content.

### *Ultrafiltration*

The determination of diffusible calcium was carried out by the method of Greenberg and Gunther (11) with minor modifications. Pyroxylin (Mallinckrodt) was used for the preparation of collodion sacs rather than Schering's collodion as advised by the above authors. In brief, the details of the procedure were as follows: 4 cc. samples of serum were ultrafiltered into Folin and Wu blood sugar tubes on a 6 unit apparatus at 150 mm. of Hg. pressure for from 3 to 4 hours, until approximately half of the serum volume had passed through the sacs. The residue in each case was transferred to a second Folin and Wu tube and then the volume of each fraction was determined by adding water from a micro burette to the 4 cc. graduation mark on the tube. Calcium was determined in each case on a 3.5 cc. aliquot of the diluted fluid, according to the procedure for total serum calcium.

The diffusible calcium, in the earlier experiments, was calculated from the calcium concentration of the ultrafiltrate by making a 5 per cent correction for protein volume. This correction would be accurate, assuming a specific protein volume of 0.75 (16), if the serum protein value was 6.7 per cent. An enormous variation in the serum protein content would be necessitated in order to cause an appreciable error due to this correction; *e.g.*, a 50 per cent variation would cause only a 2.5 per cent error in the result for diffusible calcium. In those experiments in which serum protein determinations were made, corrections for protein volume were made accordingly. No correction was made for the Donnan equilibrium effect. Even if it is a factor in ultrafiltration the correction necessary would be small—approximately 2 per cent according to Marrack and Thacker (10).

Total serum calcium, aside from the direct determination, was also estimated by summation of the calcium contents of ultrafiltrate and residue.

## 250 Diffusible and Non-Diffusible Serum Ca

The values for non-diffusible calcium recorded in Table II were determined by the difference between the diffusible calcium value and the total calcium value determined directly on the whole serum.

Before proceeding with the problem in hand a number of preliminary experiments were made to determine the efficacy of the ultrafiltration method. Although the collodion sacs were prepared by a constant procedure (17), there was an appreciable variation in

TABLE I

*Effect of Permeability of Sacs and Duration of Ultrafiltration on Diffusible Calcium Results*

Diffusible calcium is expressed as mg. per 100 cc. of serum and as per cent of total serum calcium.

Experiment 1			Experiment 3			
Volume of ultrafiltrate cc.	Diffusible Ca		Duration of ultrafiltration hrs.	Volume of ultrafiltrate cc.	Diffusible Ca	
	mg.	per cent			mg.	per cent
1.96	6.1	61.5	2	1.17	7.3	64
2.03	5.8	58.5	4	1.77	6.9	60
2.05	6.0	60.5	6	2.26	7.0	61
2.07	5.8	58.5	8	2.31	7.1	62
2.26	6.1	61.5				
Experiment 2			Experiment 4			
1.43	7.1	66	2	0.92	5.2	52.5
1.59	6.7	62.5	3	1.27	5.3	53.5
1.60	6.7	62.5	4	1.55	5.1	51.5
1.61	6.9	65	4	1.93	5.0	50.5
1.74	6.8	63.5	6	2.25	5.1	51.5
			8	2.38	5.2	52.5

permeability in the various sacs, possibly explainable by some concentration of the collodion during their preparation. Such differences were evidenced by the variation in volume of the ultrafiltrate in various samples of the same serum, the duration and pressure of ultrafiltration being constant. In such experiments small difference in permeability of the sacs did not influence the concentration of the ultrafiltrate. Table I shows the results of two experiments (Experiments 1 and 2), in each of which five 4 cc. samples of the same serum were ultrafiltered for 4 hours at the

same pressure. The conclusion that slight variations in permeability of the sacs did not influence the results was further justified by duplicate determinations made throughout this study.

Theoretically the volume of ultrafiltrate should not be a factor in the results as its concentration should be the same at all periods of the process. This was substantiated by a number of experiments in which various 4 cc. samples of the same serum were ultrafiltered for various periods of time in order to yield various volumes of ultrafiltrate. The results of two representative experiments on two different serums are shown in Table I (Experiments 3 and 4). The small variation in the results for diffusible calcium could not be correlated with the volume of ultrafiltrate. Consequently no particular care was considered necessary with respect to the volume ultrafiltered or the time involved. Usually, as previously described, the process was continued until the ultrafiltrate and residue were approximately equal in volume so that a suitable amount of solution was available for analysis.

#### EXPERIMENTAL

After withdrawal of a sample of normal blood, from 5 to 20 cc. of a 10 per cent calcium solution, as indicated in Table III, were injected intravenously in the course of from  $1\frac{1}{2}$  to 3 minutes, depending on the severity of symptoms manifested. (Usually there was a definite increase in pulmonary ventilation. Vomiting and defecation occurred in approximately half of the cases.) Blood samples were withdrawn usually at the following periods after injection, 2 to 4 minutes, 15 minutes, 30 minutes, 90 minutes, 5 hours. The blood was allowed to clot at room temperature and the serum decanted after centrifugation and kept in stoppered tubes in the refrigerator. On the following day ultrafiltration and calcium determinations were carried out. After perfecting the technique, six experiments were conducted with each of the three calcium salts (chloride, gluconate, and lactate).

#### *Results*

Due to lack of space it is not practicable to submit complete data for all experiments but Tables II to IV are sufficient to illustrate the essential points. In Table II the complete results of one representative experiment are shown. Table III records a part of

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the data of all experiments, the total serum calcium and percentage diffusible calcium for the normal serum and first and final samples after injection being sufficient to show the characteristic changes. Also the percentage of total serum calcium found on the collodion sacs after ultrafiltration, and the percentage serum protein are recorded where such determinations were made.

*Total Serum Calcium*—In all cases the first blood sample after injection showed a marked rise in total serum calcium. The level rapidly fell so that in 90 minutes it usually approximated the

TABLE II

Experiment 6, Dog 14, weight 15.2 kilos. CaCl<sub>2</sub> solution was administered intravenously (equivalent of 172 mg. of Ca). 4 cc. of serum were ultrafiltered for 4 hours in each case.

Sample withdrawn   (1)	Calcium				Ca content of fractions					Volume of fractions		
	Total direct analysis	Diffusible		Non-diffusible	Ultrafiltrate	Residue	Sacs		Total (7)+(8)	Ultrafiltrate	Residue	Loss
		(3)	(4)				(8)	(9)				
(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	
	mg.	mg.	per cent	mg.	mg.	mg.	mg.	per cent	mg.	cc.	cc.	per cent
Before injection	9.9	5.5	56	4.4	3.4	6.2	0.4	4	10.0	2.31	1.65	1
After injection												
4 min.	15.4	8.1	53	7.3	4.8	8.8	2.0	12.5	15.6	2.21	1.71	2
15 "	13.4	6.8	51	6.6	4.3	7.5	1.7	12.5	13.5	2.37	1.61	0.5
30 "	12.5	6.4	51	6.1	3.8	7.6	1.0	8	12.4	2.25	1.72	1
90 "	11.0	6.3	57	4.7	3.7	6.7	0.5	4.5	10.9	2.22	1.72	1.5
5 hrs.	9.5	5.3	56	4.2	3.4	6.1	0.4	4	9.9	2.40	1.54	1.5

normal value and after 5 hours was usually as low or lower than the original level.

*Calcium Fractions*—The diffusible calcium increased in each case with the total serum calcium and likewise returned to normal with it as shown in Column 3, Table II. However, the rise in total serum calcium could never be accounted for by the rise in this fraction, in spite of the fact that the calcium injected was in a form entirely diffusible. This fact is brought out in Tables II and III, by the figures showing percentage diffusible calcium of total calcium. After calcium injection the proportion of diffusible

calcium is not markedly changed, showing that the rise of total serum calcium is the result of increases in both fractions. To illustrate, if the rise in total serum calcium in the 4 minute sample,

TABLE III  
*Summary of Results*

Experiment No.	Weight of dog	Solution injected*	Normal serum				2-4 min. after injection				5 hrs. after injection			
			Total Ca	Diffusible Ca	Ca on sacs	Serum protein	Total Ca	Diffusible Ca	Ca on sacs	Serum protein	Total Ca	Diffusible Ca	Ca on sacs	Serum protein
Calcium chloride														
	kg.	cc.	mg.	per cent	per cent	per cent	mg.	per cent	per cent	per cent	mg.	per cent	per cent	per cent
1	11.4	5	12	61.5			17	55.5			10	59		
2	9.5	5	11	70			18	61			9	65		
3	9.5	10	11	64			21	55			9	70		
4	15.4	5	12	54			15	51			9	53.5		
5	15.4	10	11	58.5			17	53.5			11	59.5		
6	15.2	172 mg. Ca	10	55	4		15	52	12.5		10	55	4	
Calcium gluconate														
7	8.2	20	10	58			21	38			11	57.5		
8	8.2	20	10	61.5			23	54.5			11	62.5		
9	6.8	20	11	60.5			30	58.5			12†	55.5†		
10	6.8	10	9	62.5			18	61			10‡	61‡		
11	15.0	19	11	55.5			16	46.5			10	58		
12	15.0	172 mg. Ca	10	61	3		17	59	8.5		9	60	8	
Calcium lactate														
13	15.0	172 mg. Ca	10	58.5	3		14	52.5	10		10	51.5	4	
14	10.5	8	11	59	7		12	54.5	14		9	60	5	
15	15.1	18	11	52.5	1	8.1	18	55.5	6	7.8	11	50	1	7.3
16	14.5	20	11	50.5	0	6.55	18	50	7	6.75	11	44	3	5.9
17	19.0	20	10	58	0.5	7.8	17	44.5	10	7.8	10	52.5	2	6.9
18	10.1	20	10	61.5	2.5	6.3	20	55	12	6.4	10	58	6	5.8

\* A 10 per cent calcium solution was injected in all cases except in Experiments 6, 13, and 14, in which 3, 7, and 5 per cent solutions respectively were used.

† 3 hours after injection.

‡ 24 hours after injection.

Table II, was entirely due to a rise in diffusible calcium, the percentage diffusible calcium would be 71 instead of 52 per cent.

The readjustment of relationship is explainable by an equilibrium between calcium and serum protein and such results are in keeping with the conclusions of those workers mentioned previously (5, 8-10). This also infers that the normal serum protein is not saturated with calcium but in the presence of greater calcium concentrations is capable of combining with more than the normal amount.

The increase in non-diffusible calcium on the addition of diffusible calcium salt solutions is not dependent on an increase in serum protein. This is clearly demonstrated by those experiments in

TABLE IV  
*Addition of Calcium to Serum in Vitro*

Sample	Experiment 1			Experiment 2				Experiment 3			
	Total Ca		Diffusible Ca	Total Ca		Diffusible Ca	Ca on sacs	Total Ca		Diffusible Ca	Ca on sacs
	mg.	mg.		mg.	mg.			mg.	mg.		
Normal serum .....	10.6	6.9	65	9.4	6.5	69	2	10.4	6.8	65	1
Serum + Ca, Sample I .....	18.2	10.9	60	14.4	9.4	65	9	20.2	12.8	63	8
Serum + Ca, Sample II .....	27.9	15.8	57	24.2	13.7	56	5	39.2	22.9	58	8.5

which serum protein determinations were made and recorded in Table III. The serum protein did not rise after the injection and in fact often showed a definite decrease in the final samples, which is explainable by the removal of an appreciable volume of blood in the course of an experiment.

The increase in non-diffusible calcium is a function of the serum alone, and not due to any interchange between blood and tissues. This was shown by certain *in vitro* experiments in which 1 cc. of calcium chloride solutions of concentrations sufficient to cause a definite rise in serum calcium was added to 25 cc. samples of serum. To the normal or control sample was added 1 cc. of distilled water to make a similar dilution. All samples were immediately ultrafiltered at the same time. The results of three such

experiments are recorded in Table IV. Here, again, the percentage of diffusible calcium is not increased, necessitating a proportionate increase in the non-diffusible fraction.

With the experimental procedure used it is difficult to determine the rate of readjustment described. It is evident in blood samples withdrawn from 2 to 4 minutes after calcium administration and also in serum, *in vitro*, when ultrafiltration is begun immediately after the addition of calcium chloride. However, the process of ultrafiltration usually takes from 3 to 4 hours, during which period at least a part of the fractions are in contact.

If the figures showing the percentage diffusible calcium in Tables III and IV are examined more closely, it is observed that there is actually a tendency for them to decrease in the presence of a high serum calcium content. In the *in vitro* experiments (Table IV) this occurs quite regularly. In Table III (*in vivo* experiments) the percentage diffusible calcium in the first sample after injection is lower than before injection in every case except one (Experiment 15). Furthermore in the final example there is a tendency for the percentage diffusible calcium to return to normal. In only four experiments (Experiments 9, 13, 15, and 16) is the figure higher in the first sample after injection than in the final sample. Such differences are in many cases small and within the limit of experimental error which we consider to be approximately  $\pm 2.5$  per cent. However, the regularity of the changes with respect to direction suggests some significance, and is possibly explainable by the findings mentioned below.

#### *Calcium Content of Collodion Sacs*

In the earlier experiments it was noted that the sum of the calcium contents of ultrafiltrate and residue fell short of the total serum calcium value. The apparent loss of calcium during ultrafiltration was especially noticeable in serums with a high calcium content. The reason for this discrepancy was disclosed by analyzing the collodion sacs for calcium, which became a routine procedure in the latter part of the work. Small amounts of calcium such as those found when using normal serums might be expected on analysis of the sacs, as it was impossible to rid them of all traces of ultrafiltrate and residue. The sum of the volumes of ultrafiltrate and residue was always less than that of the original serum,



but this loss in volume was rarely greater than 3 per cent of the 4 cc. ultrafiltered and was usually less. However, in those cases involving serums of high calcium content the calcium contents of the sacs were too great to be explained in this way—in certain cases amounting to 12 or 14 per cent of the total serum calcium, as recorded in Table III. This observation suggests a partial precipitation of serum calcium in the presence of high calcium concentrations, such a precipitate being adsorbed on or filtered out by the sacs in the process of ultrafiltration.

If it is assumed that the calcium adherent to the sacs after ultrafiltration comes from the diffusible fraction, then an explanation is provided for the apparent lowering of the percentage of diffusible calcium when the total calcium content is increased. This assumption is in keeping with two *in vitro* experiments which for some unknown reason proved exceptions to the general rule in that there was no adsorption of calcium on the sacs when the calcium content of the serum was increased by the addition of calcium chloride. In these experiments the percentage of diffusible calcium was approximately the same both before and after the increase in total calcium content.

In a series of experiments undertaken to explain the high calcium content of the sacs, it was found that no precipitate could be centrifuged from serums which showed this phenomenon on ultrafiltration. This was to be expected as Hastings, Murray, and Sendroy (18) have demonstrated that serum, in which the calcium content had been doubled by the addition of calcium bicarbonate, showed no precipitation of calcium even when equilibrated with solid calcium carbonate for 22 hours. It was then conceived that a precipitation or adsorption of calcium might be a direct result of ultrafiltration, in which process the concentration of serum protein and the calcium-protein compound of the fluid within the sac is gradually increased to twice that which previously existed. However, it was found that the adsorption did not depend on ultrafiltration. Serums allowed to stand in the sacs for varying periods of time showed a gradual loss of calcium to the sacs similar to the loss in a parallel series which was ultrafiltered for the same periods.

At present, neither the cause of the adsorption nor the form in which the calcium is adsorbed is known. Whatever may be the explanation, our conclusion is not affected with respect to the approximately proportional increase of both diffusible and non-

diffusible calcium following the administration of diffusible calcium salts.

#### SUMMARY AND CONCLUSIONS

1. When the serum calcium content of normal dogs is increased by intravenous injection of wholly diffusible calcium salts, the relationship between diffusible and non-diffusible calcium assumes approximately normal proportions, explainable by an equilibrium between calcium and serum protein. This indicates that the normal serum protein is not saturated with calcium but under the above conditions is capable of combining with more than the normal amount.

2. The above finding is obtained *in vitro* by the addition of calcium chloride to blood serum, showing that such a readjustment of equilibrium is a function of the serum itself and not dependent on any interchange between blood and tissues.

3. In the presence of high serum calcium values the percentage of diffusible calcium determined by ultrafiltration is even slightly lower than the normal. Under the same conditions the collodion sacs after ultrafiltration show an appreciable calcium content, suggesting a partial adsorption of diffusible calcium during the process.

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## CARBOHYDRATE CHANGES DURING ANAEROBIOSIS OF MAMMALIAN MUSCLE

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Fletcher and Hopkins (1) were the first to show definitely that lactic acid accumulates when frog muscle is kept without oxygen. Parnas and Wagner (2) investigated the changes in glycogen and other carbohydrates under experimental conditions closely similar to those chosen by Fletcher and Hopkins. They were thus able to correlate their results with those obtained by the latter authors. After anaerobic tetanic stimulation the disappearance of glycogen was of the same order of magnitude as the accumulation of lactic acid. On the other hand, after resting anaerobiosis, no diminution of the glycogen content was observed, so that the accumulation of lactic acid in Fletcher and Hopkins' experiments remained unaccounted for. Laquer (3), who determined glycogen and lactic acid in minced frog muscle after incubation in phosphate buffer, also failed to find a strict quantitative relationship between the changes in these two substances. Meyerhof (4) found that under all conditions investigated by him (*i.e.*, tetanic or single shock stimulation in  $N_2$ , resting anaerobiosis, chemical or heat contraction) there existed a satisfactory parallelism between carbohydrate and lactic acid changes in frog muscle. Two balance experiments were made on intact resting muscle during anaerobiosis, one conducted at  $14^\circ$  for 45 hours, the other at  $22.5^\circ$  for 6 hours. The results (in mg. per 100 gm. of muscle) were as follows:

	$14^\circ$	$22.5^\circ$
Total carbohydrates which disappeared.....	375	150
Lactic acid accumulated.....	262	120
Balance.....	-113	-30

It will be noted that only 70 and 80 per cent of the total carbohydrates which disappeared (mainly glycogen) were recovered as

lactic acid. In four experiments on minced muscle 101, 105, 81, and 97 per cent were recovered as lactic acid. For intact muscle at least, an accumulation of intermediaries (other than simple sugars) was not excluded by these experiments. While this work was in progress, Kerly (5) published balances on minced frog muscle suspended in phosphate buffer and kept for 3 hours at 37°. In a series of experiments on autumn frogs on an average 645 mg. of glycogen disappeared and 626 mg. of lactic acid accumulated; in summer frogs these figures were 538 and 606 mg. per cent. In bicarbonate buffer more glycogen disappeared than lactic acid accumulated. No provisions were made to exclude oxygen in these experiments.

For mammalian muscle this problem has been approached by Macleod and his coworkers. Simpson and Macleod (6) froze cat muscle, pulverized it in the frozen state, and allowed it to thaw. More glycogen disappeared than lactic acid accumulated. Fraser and Macleod (7) determined glycogen and lactic acid in muscle immediately and 1 hour after killing rabbits by chloroform. In eight out of twelve experiments the glycogen rose (10 to 340 mg. per cent); in four it fell (20 to 130 mg. per cent). Irrespective of fall or rise in glycogen, the lactic acid rose, the increase ranging from 20 to 230 mg. per cent. In their "balance" experiments Anderson and Macleod (8) killed rats by spinal transection and determined glycogen, free sugar, and lactic acid in one leg immediately and in the other leg 1 hour after death of the animal which was kept at room temperature. Immediately after death the muscle contained 188 to 434 mg. per cent of lactic acid; a rise of 13 to 186 mg. per cent occurred 1 hour after death. At the same time the glycogen either rose (21 and 58 mg. per cent) or fell (17 to 64 mg. per cent). The changes in free sugar were slight. Since the average glycogen content of the muscles taken immediately after death was 362 mg. and the average lactic acid content 291 mg. per cent, the combined glucose value of both amounted to 653 mg. per cent. This, to the authors "is so high as to suggest some unknown error in the determination." Actually, this value is close to the glycogen value found in rat muscle removed from the living animal under conditions which exclude glycogenolysis (9).

In spite of the occurrence of glycogenolysis in these rats killed by spinal transection, in twelve out of fifteen experiments the glycogen content of the legs, when both were taken for analysis

immediately after death, showed good agreement. Equality of glycogen in symmetrical muscles cannot be relied upon to prove absence of glycogenolysis because it may proceed at a fairly equal rate on both sides.

In another series of experiments by Anderson and Macleod rats were kept under luminal anesthesia and the muscles of one leg frozen *in situ*; the other leg was cut off and kept for 1 hour at room temperature. In five out of six experiments the glycogen fell and in all of them the lactic acid rose. There was no quantitative correlation between these two changes. The lactic acid content of the frozen muscles ranged from 49 to 216, average 134 mg. Davenport and Davenport (10) who introduced this technique obtained much lower and less variable values for resting lactic acid in several mammalian species. The same is true for the experiments of Martin and collaborators (11) on dog muscle. Anderson and Macleod state that their methods cannot "be counted upon to give an accurate picture of the behavior of both glycogen and lactic acid in muscle after death." Nevertheless, they feel justified in concluding that glycogen of intact mammalian muscle does not diminish within 1 hour after death. Since they found that lactic acid accumulates under these conditions, they assume that it is derived from a substance other than glycogen, possibly of the nature of a trisaccharide.

If these conclusions had any basis in fact, a qualitative difference would exist between mammalian and amphibian muscle. This possibility seemed important enough to suggest a reinvestigation. The more so, since one intermediary in the breakdown of glycogen to lactic acid, namely hexosemonophosphate, could be included in the balance (12).

#### EXPERIMENTAL

Both gastrocnemii were removed from the living animal as described in a previous paper (9). One muscle was analyzed immediately, while the other was kept for various lengths of time at 37° in a moist chamber through which N<sub>2</sub> was passing.<sup>1</sup> The lactic acid values for resting muscle (Table IV) indicate that

<sup>1</sup> Anderson and Macleod state that their experiments were carried out at room temperature. In dealing with tissues of warm blooded animals it seemed preferable to us to conduct the experiments at body temperature.

removal of the muscle from the body was accomplished without causing appreciable glycogenolysis.

All experiments were performed on rats previously fasted for 24 hours. Since glycogen, hexosephosphate, and lactic acid could not be determined in one and the same muscle, a separate series of experiments was made for each of these constituents.

The glycogen content of the right gastrocnemius muscle of the rat agrees closely with that of the left (9). In the present paper a comparison of the right and left side was made under the following conditions. One muscle, immediately after its removal, was submerged in cold KOH contained in a tared and stoppered 15 cc. centrifuge tube. The tube was weighed and placed in the boiling water bath. The other muscle was weighed on a watch-glass as

TABLE I

*Influence of Cold and Hot KOH on Glycogen Determination in Right and Left Gastrocnemius of Rat*

The values are given in mg. per 100 gm. of muscle.

Cold KOH (Right)	Hot KOH (Left)
540	553
508	466
546	545
556	549
538	528

quickly as possible and then submerged in hot KOH. 1 cc. of 60 per cent KOH per gm. of muscle was used in both cases. Table I shows that no difference was found between cold and hot KOH; the former was used in all actual experiments. The tubes containing muscle and KOH were kept for 3 hours in the water bath with occasionally shaking. Evaporation was prevented by means of a small funnel. After cooling, the contents of the tubes were diluted with 1 volume of water and absolute alcohol was added to give a 70 per cent concentration. The total volume was thus 12 to 16 cc.

Kerly (13) has shown that it is desirable to apply a correction for the slight solubility of glycogen in aqueous alcohol when one is dealing with very small quantities. In the present work 1 to 7

mg. of glycogen were estimated. Since a solubility of only 0.015 mg. in 10 cc. of 70 per cent alcohol could be calculated from Kerly's data, a correction was not applied.

When a quantity of glycogen,<sup>2</sup> approximately one-half the amount present in one rat gastrocnemius, was precipitated with 66 to 80 per cent alcohol (total volume 14 cc.), practically the same values were obtained (Table II). The fact that a seemingly larger yield of glycogen is obtained in tissue analysis (for instance liver, Lawrence and McCance (15)), when 80 instead of 66 per cent alcohol is used is mostly due to the presence of more impurities in the glycogen precipitate when the more concentrated alcohol is used. These impurities are not removed by washing and have a

TABLE II

*Influence of Varying Alcohol Concentrations on Yield of Glycogen*

2 cc. of a glycogen solution containing 2.50 mg. of glycogen were precipitated in a volume of 14 cc.

Percentage of alcohol	Benedict method	Hagedorn-Jensen method
	mg.	mg.
66	2.47	2.56
70	2.45	2.56
70	2.45	2.58
75	2.51	2.56
80	2.49	2.56
80	2.45	2.54

marked influence on sensitive micro sugar methods. That this is actually the case has been found by means of yeast fermentation. The amount of non-sugar reducing substances was perceptibly higher when the muscle glycogen was precipitated with 80 instead of 70 per cent alcohol.

After standing overnight the glycogen precipitate was centrifuged off and washed on the centrifuge twice with 2 cc. each of 70 per cent, once with 95 per cent alcohol, absolute alcohol, ether, and again absolute alcohol. In all cases the glycogen, after sufficient agitation with a glass rod, appeared in fine dispersion and was of

<sup>2</sup> The glycogen was extracted from rabbit liver by means of trichloroacetic acid as recommended by Rona and van Eweyk (14) and subsequently purified by repeated precipitations with alcohol.



a light grayish color. If it is brown and sticky or gummy, it has not been washed successfully. After driving off traces of alcohol-ether, 1.5 cc. of 0.6 N HCl were added and the tubes kept for 3 hours in the water bath. The time required for acid hydrolysis under the conditions of the present experiments was tested on a sample of purified glycogen. 3 hours were found to be the optimal period, as originally claimed by Pflüger.

After hydrolysis the contents of the tube were washed into a volumetric flask (10 to 25 cc.) and neutralized against litmus by adding, drop by drop, a NaOH solution (about 3 per cent). After the volume was made up, the contents were filtered and 1 to 2 cc. taken for analysis. Benedict's (16) method was used, and 0.2 to 0.3 mg. of glucose determined in most experiments. In order to test for the presence of non-sugar reducing substances the following procedure was followed: 10 cc. of glycogen hydrolysate were added to washed yeast and kept at room temperature for 15 minutes (yeast plus 10 cc. of  $H_2O$  and yeast plus 10 cc. of 0.025 per cent glucose solution were run simultaneously). After centrifugation the supernatant fluid was used to dilute 1 cc. of a 0.2 per cent glucose solution to 10 cc. The glycogen hydrolysates were practically free of non-sugar reducing substances capable of reacting with the Benedict reagent. (Under the same conditions there was always some non-sugar reducing material present which reacted with the Hagedorn-Jensen method.)

For lactic acid determination, the muscle was introduced immediately after extirpation (or after incubation in  $N_2$ ) into a tared tube containing 10 cc. of ice-cold 2 per cent HCl. After 1 to 2 minutes the tube was weighed, the contents transferred to a mortar, and the muscle was crushed with the pestle in the cold acid and ground with sand to a fine pulp. The proteins were precipitated by addition of 5 cc. of 5 per cent  $HgCl_2$ , as recommended by Schenk (17). For convenience, the final dilution was made 1:10 by addition of water, it being assumed that the water content of the muscle was 80 per cent. After 10 minutes standing the solution was filtered, followed by precipitation of the excess mercury with  $H_2S$  and removal of  $HgS$  and  $H_2S$  in the usual manner. An aliquot part of the final filtrate was neutralized and precipitated with copper sulfate and lime. The lactic acid content was determined according to the method of Friedemann, Cotonio, and Shaffer

(18), the modification of Wendel<sup>3</sup> being used. An average of 97.7 per cent (94.1 to 100.8 per cent) was recovered with this procedure when recrystallized zinc lactate was analyzed in quantities comparable to the amounts encountered in the present work. No correction was applied to the glycogen or lactic acid values. The loss of glycogen during hydrolysis in 0.6 N HCl (about 3 per cent according to Nerking (19)) is approximately balanced by the incomplete recovery of lactic acid as shown above. Hexosemono-

TABLE III

*Glycogen Content of Rat Muscle Kept Anaerobically at 37°*

All values are given in mg. per 100 gm. of muscle.

The figures in parentheses indicate disappearance of glycogen.

Time of incubation			
0 min.	30 min.	60 min.	120 min.
589	518(71)		
540	424(116)		
501	420(81)		
545	402(143)		
472	352(120)		
456		253(203)	
498		251(247)	
486		214(272)	
620		296(324)	
624		395(229)	
505			57(448)
565			82(483)
531			113(418)
510			99(411)
571			61(510)
Average . . . 534	425(109)	282(252)	82(452)

phosphate was determined according to Cori and Cori (12). All experiments were carried out during July and August, 1931.

On plotting the average values of Tables III and IV it was found that the disappearance of glycogen and accumulation of lactic acid were almost linear functions of time from 0 to 60 minutes. During the following 60 minutes there was a slowing up of both

<sup>3</sup> Personal communication, since published (Wendel, W. B., *J. Biol. Chem.*, **94**, 717 (1931-32)).

processes. After 2 hours in  $N_2$  at  $37^\circ$  the muscles were always in rigor and the glycogen stores were nearly exhausted. It is possible that the "lactic acid maximum" was reached before 2 hours and that no further accumulation of lactic acid occurred during the latter part of the 2 hour period. Muscles under these conditions remain irritable to direct faradic stimulation for about 40 minutes.

After 30 minutes of anaerobiosis the hexosephosphate content had hardly changed (Table V). After 1 hour there was a definite increase which persisted during the next hourly interval. In muscle kept for 2 hours in  $N_2$  the P found was much higher than the P calculated for the Embden monoester. This might have

TABLE IV

*Lactic Acid Content of Rat Muscle Kept Anaerobically at  $37^\circ$*

All values are given in mg. per 100 gm. of muscle.

The figures in parentheses indicate average accumulation of lactic acid.

Time of incubation			
0 min	30 min.	60 min.	120 min.
14	96	224	374
17	100	227	432
19	106	233	477
28	116	246	504
29	140	255	514
31			531
Average... 23	112(89)	237(214)	455(432)

been due to the accumulation of hexosediphosphate of the Harden-Young type, but hydrolysis curves of the trichloroacetic acid extract of these muscles in  $N$  HCl at  $100^\circ$  gave no indication of it. It was found, however, that the fraction which is split off during 7 minutes of hydrolysis (Lohmann's pyrophosphate) was very low (6, 14, and 15 instead of 42 mg. per cent as in fresh muscle). This made it probable that adenylic (or inosinic) acid was formed from adenosinetriphosphate during anaerobiosis and since these compounds form soluble barium salts, they would be included in the hexosemonophosphate fraction as separated in the course of the method and would increase the P content of that fraction without appreciably affecting the reducing value. The

outcome of pentose reactions strengthened this supposition. The hexosemonophosphate fraction from fresh muscle gave hardly any pentose reaction (orcinol or phloroglucinol test) whereas the reaction was strongly positive in muscle kept in  $N_2$  for 120 minutes. The fact that the P found for the 2 hour period (Table V) is much higher than the P calculated for a hexosemonophosphate is therefore ascribed to the presence of inosinic (or adenylic) acid formed during anaerobiosis.

The present experiments show that once mammalian muscle is excised, the rate of glycogen breakdown becomes comparatively

TABLE V  
*Hexosephosphate Content of Rat Muscle Kept Anaerobically at 37°*  
All values are given in mg. per 100 gm. of muscle.

Time of incubation											
0 min.			30 min.			60 min.			120 min.		
Hexose	P found	P calculated	Hexose	P found	P calculated	Hexose	P found	P calculated	Hexose	P found	P calculated
			45.3	11.5	7.8	77.4	17.0	13.3	85.8	23.1	14.8
			53.5	10.2	9.2	87.8	16.6	15.1	90.0	28.4	15.5
			69.6	14.3	12.0	103.6	20.1	17.8	98.1	29.1	16.9
53.3*	8.2	9.2	56.1	12.0	9.7	89.6	17.9	15.4	91.3	26.9	15.7

\* Average of seven experiments (12).

slow. From Tables III and IV it can be calculated that per minute (at 37°) less than 4 mg. per cent of glycogen disappears or lactic acid accumulates.<sup>4</sup> Hence there is little purpose in freezing the excised muscle, as is sometimes done in glycogen or lactic acid analysis. The few seconds, or even 1 minute, which elapse before glycogenolysis is stopped by placing the muscle in concentrated alkali or cold acid play no rôle. No method has as yet been devised by means of which an animal could be killed without causing

<sup>4</sup> While this article was in press, Haldi (20) showed that lactic acid accumulation in dog muscle kept outside the body at 38° for 1 to 12 minutes follows a straight line curve; 5 mg. per cent of lactic acid per minute were formed.

rapid glycogen breakdown. The high lactic acid content of muscle of rats killed by spinal transection (8) or the high hexosephosphate content of muscle of rats killed by bleeding while under amytal narcosis (21) shows that these procedures form no exception. If the preservation of *in vivo* conditions is aimed at, the muscle should therefore be extirpated from the living anesthetized animal.

When the changes in glycogen, lactic acid, and hexosemonophosphate occurring in rat muscle kept in  $N_2$  at  $37^\circ$  are balanced against each other, the glucose values of these substances being used, it is found that on an average in 30 minutes 109 mg. of glycogen disappear and 93 mg. of lactic acid plus hexosephosphate accumulate; in 1 hour 252 mg. disappear and 252 mg. accumulate; in 2 hours 452 mg. disappear and 472 mg. accumulate. The changes in hexosephosphate play but a slight rôle for the balance. If any other intermediary between glycogen and lactic acid should accumulate under these conditions, or if a substance other than glycogen should serve as a source of lactic acid, these balances hardly give an indication of it. The finding of Macleod and collaborators that lactic acid accumulates in intact mammalian muscle after death without a corresponding decrease in glycogen could not be confirmed.

#### SUMMARY

1. Glycogen, lactic acid, and hexosemonophosphate were determined in right and left gastrocnemius muscles of rats, extirpated from anesthetized animals. One muscle was analyzed immediately and one after a period of survival in  $N_2$  at  $37^\circ$ .

2. The disappearance of glycogen on the one hand, and accumulation of lactic acid plus hexosephosphate on the other hand, nearly balanced each other after 30, 60, and 120 minutes of anaerobiosis.

3. Hydrolysis of trichloroacetic acid extracts of muscle in  $N$  HCl at  $100^\circ$  gave no indication of an accumulation of hexosediphosphate (Harden-Young ester) during anaerobiosis. The organic phosphate fraction hydrolyzed during 7 minutes decreased markedly, indicating a conversion of adenosinetriphosphate to adenylic (or inosinic) acid under this condition.

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## THE OSMOTIC BEHAVIOR OF WATER OF BLOOD SERUM

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In recent years the physicochemical state of water in biocolloidal solutions has aroused much interest owing largely to the theories which have been formulated to explain the regulation of certain phenomena in living organisms, such as regulation of temperature, edema, winter hardiness of insects and plants, etc. Much discussion and controversy have arisen as to whether part of the water present in a colloidal solution is held with the solute in such a way as to behave differently from the remaining water. Kuhn (1), following Ostwald (2), has indicated that in considering the state of water in colloidal materials, the water may be described as existing in several different forms, such as occlusion water, adsorption water, capillary water, colloiddally bound water, chemically bound water, osmotic water, and complexly bound water, and that one or more different methods have been utilized to determine quantitatively each of these various classes of water described. Certainly some of the controversies in the literature may be traced to failure to differentiate clearly between these types of water in colloidal solution and especially to the loose usage of the terms "free" and "bound" water.

Gortner (3) has limited his usage of the term free water to water which exhibits a normal depression of the freezing point upon dissolving substances added to it, while bound water is an associated form of water in which the dissolving power is assumed to be zero. Newton and Gortner (4) have obtained evidence that in various plant saps added solute gives abnormally great depression of the

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freezing point. They interpret this to mean that part of the water is bound.

Hill (5) has recently studied the state of water in muscle and blood. His definitions of the terms free and bound water are analogous to Gortner's. By means of his vapor pressure method he has shown that the so called free water of blood was approximately equal to the total water content, "being perhaps two per cent less." Furthermore, contrary to Overton's conclusions, Hill's studies would indicate that within the 2 per cent error of his method all of the water of muscle is free.

Neuhausen (6) concluded from a comparison of the osmolar concentration of dog serum obtained from chemical analyses given in the literature (assuming an arbitrary degree of dissociation) with the osmolar concentration calculated from the lowering of the vapor pressure that the total water of serum is osmotically active. The meaning of the term bound water when used by Neuhausen is different from its meaning as used by Gortner and by Hill. According to Neuhausen, bound water cannot be measured by any of the colligative properties since the bound water includes the water imbibed by protein even though it be capable of dissolving salts in the same concentration and with the same freezing point depression as the remaining water in the serum. In Gortner's terminology bound water would not be capable of dissolving salts. Neuhausen's estimations would, therefore, lend support to the view that the water in the serum is present in the free state in the Gortner sense.

In our present studies we have concerned ourselves principally with the extent to which one of the colligative methods, namely the freezing point, may be used to determine changes in the osmotic behavior of water in blood serum. It is in the sense of the definitions of free and bound water used by Gortner and by Hill that these studies were undertaken. They have not been designed to consider such factors as the water of imbibition, colloiddally bound water, etc., except as these latter types affect the osmotic behavior of the total water present.

### *Methods*

Our method of determining the state of water in blood serum is in its general application similar to that employed by Newton and

Gortner in their studies on plant juices. The depression of the freezing point ( $\Delta t_{\text{obs.}}$ ) is determined in a portion of blood serum alone and also in portions of the same serum to which a solute such as sucrose, urea, or NaCl has been added. By measuring the total solid and water contents of the serum, the amount of solute added, and by determining the depression of the freezing point of pure solutions containing the solute in the same concentration in water, it is then possible to calculate a theoretical freezing point depression ( $\Delta t_{\text{calc.}}$ ). This theoretical depression of the freezing point is equal to the freezing point depression of the serum alone plus the freezing point depression of a pure solution of the solute in the same concentration, in water. If the total water in the serum plus solute system behaves as solvent, the calculated depression of the freezing point should equal the observed, but if some of the water is associated in such a way as to prevent its acting as a solvent, the freezing point lowering should be greater than the calculated.

The freezing point of the solutions studied was determined by the method of Stadie and Sunderman (7). The method was modified by the use of an especially constructed Heidenhain thermometer. This thermometer differs from the ordinary type in that the capillary extends down to within  $2\frac{1}{2}$  inches of the bulb so that the bulb and bulb stem are immersed at the freezing temperature. With the use of the ordinary Heidenhain thermometer in fluctuating room temperatures, considerable error may result from the variations in the stem correction since the bulb stem has a much larger diameter than the capillary scale. The range of the scale extends over  $4^{\circ}$  with markings at  $0.01^{\circ}$  intervals. Included in the case of the thermometer is a narrow "*Faden*" thermometer placed in the mid-section in order to estimate the mean stem temperature. During the manufacturing the thermometer was pointed at  $0.5^{\circ}$  intervals. The thermometer has been checked at every  $0.2^{\circ}$  and  $0.3^{\circ}$  interval over the first part of the working range by the Bureau of Standards. When the thermometer was not in use in the freezing point apparatus, the bulb and bulb stem of the thermometer were kept immersed in finely shaved ice contained in a thermos flask and the capillary was exposed to the room temperature. The thermometer was thus kept practically always under the same conditions of temperature,

never being more than  $\pm 2^\circ$  from the zero point. All readings on this thermometer scale were made with a cathetometer. The freezing point measurements of standard solutions, such as of KCl and NaCl, were within  $\pm 0.002^\circ$  of the smoothed curve data of Stadie and Sunderman.

Specific gravity of the serum was obtained by the use of a 10 cc. pycnometer. The total solids were determined by drying at  $100^\circ$  weighed amounts of the solutions to constant weight. Chloride measurements of the serum were made by means of the Wilson and Ball (8) modification of the Van Slyke method.

In the preparation of the solutions studied, conductivity water was used. The reagents were recrystallized two or more times. All solutions were made up by weight and the concentrations have been expressed in terms of molality ( $N_w$ ) number of gram-formula weights per kilo of water).

### Results

#### *Effect of Added Sucrose on Freezing Point Depression of NaCl and Blood Serum*

*Freezing Points of Sucrose Solutions*—The freezing points ( $\Delta t_{\text{obs.}}$ ) of ten different solutions of sucrose in water ranging in concentrations from 0.0645  $N_w$  to 0.983  $N_w$  were determined. By plotting molecular freezing point depression ( $\lambda$ ) against the molality ( $N_w$ ) a straight line relationship may be obtained which may be expressed by the formula

$$\lambda = 1.858 + 0.204 (N_w)$$

where  $\lambda = \frac{\Delta t}{N_w}$ .

Similar data of the molecular freezing point depression of sucrose solutions were obtained by Rivett (9).

*Freezing Point of Sucrose and Sodium Chloride in Water*—In order to determine whether the activity of electrolytes, such as are present in serum, is influenced by the addition of a non-electrolyte, weighed amounts of sucrose were added to a known solution of sodium chloride. In these solutions the degree of lowering of the freezing point due to both constituents may be calculated independently ( $\Delta t_{\text{calc.}}$ ). From the data obtained from this system and given in Table I the average mean ratio of the observed freez-

TABLE I

*Freezing Point Data of NaCl Solutions, Normal Dog Serum, and Febrile Dog Serum with Added Sucrose*

Solution	$C_{15}H_{22}O_{11}$	$\Delta t_{obs.}$	$\Delta t_{C_{15}H_{22}O_{11}}^*$	$\Delta t_{calc.}$	$\frac{\Delta t_{obs.}}{\Delta t_{calc.}}$
	$N_w$ per kg. $H_2O$	$^{\circ}C.$	$^{\circ}C.$	$^{\circ}C.$	
0.1605 $N_w$ NaCl		0.558			
Same + sucrose	0.0597	0.675	0.112	0.670	1.007
	0.1010	0.750	0.190	0.748	1.003
	0.1184	0.781	0.223	0.781	1.000
	0.1812	0.897	0.344	0.902	0.995
	0.2015	0.942	0.383	0.941	1.001
	0.2360	1.007	0.450	1.008	0.999
	0.2950	1.122	0.566	1.124	0.998
	0.302	1.137	0.580	1.138	0.999
	0.510	1.554	1.000	1.558	0.996
Average.....					1.000
0.0533 $N_w$ NaCl + su- crose		0.190			
	0.500	1.170	0.980	1.170	1.000
	0.7485	1.691	1.505	1.695	0.997
	0.993	2.237	2.042	2.232	1.002
Average.....					0.9997
Normal dog serum†		0.578			
Same + sucrose	0.0403	0.654	0.075	0.653	1.002
	0.1364	0.834	0.257	0.835	0.998
	0.2263	1.012	0.432	1.010	1.002
	0.3548	1.275	0.687	1.265	1.007
Average.....					1.0022
Febrile dog serum‡		0.591			
Same + sucrose	0.1086	0.796	0.204	0.795	1.001
	0.2053	0.987	0.390	0.981	1.006
	0.482	1.548	0.943	1.534	1.009
Average.....					1.005

\* Calculated from equation,  $\lambda = 1.858 + 0.204 (N_w)_{sucrose}$ .

† The specific gravity of the serum was  $1.02087 \left( \frac{20^{\circ}}{20^{\circ}} \right)$ ; the total water content was 909.98 gm. per kilo of serum.

‡ The specific gravity was  $1.0271 \left( \frac{20^{\circ}}{20^{\circ}} \right)$ ; the total water content was 911.69 gm. per kilo of serum.

ing point depression to the calculated was 0.999, from which we may conclude that, within the limit of error of our measurements, sucrose and sodium chloride in solution with water obey the principle of additivity. Sucrose added to potassium chloride solutions showed the same additivity in the lowering of the freezing point.

*Freezing Points of Normal Serum and Normal Serum with Sucrose Added*—500 cc. of blood were removed from a normal dog by cardiac puncture. The blood was defibrinated, centrifuged, and the serum removed under oil. In Table I are given data of the observed depressions of the freezing point ( $\Delta t_{\text{obs.}}$ ) of this serum and of the serum plus sucrose. The calculated lowerings of the freezing point in the specimens containing serum plus sucrose were obtained by adding the freezing point depression of the serum to the calculated freezing point depressions of solutions containing sucrose in the same concentrations in water ( $\Delta t_{\text{calc. C}_{12}\text{H}_{22}\text{O}_{11}}$ ). The ratios of the observed to the calculated freezing points are shown to be unity within the limit of the error of our measurements. This is in accord with the additivity to be expected if the total water of the serum were to dissolve the sucrose; i.e., if the total water of the serum behaved in an osmotically normal manner.

#### *Effect of Added NaCl on Freezing Point Depression of Blood Serum*

In order to determine whether an added portion of an electrolyte normally present in serum is capable of being dissolved in the total water of serum, freezing point measurements were also made on ox serum with added NaCl. Since the degree of dissociation of NaCl decreases considerably with increasing concentration, the total NaCl concentration of these solutions (ox serum plus NaCl) was calculated on the assumption that the chloride normally present in serum exists entirely as NaCl. The results of these studies are given in Table II in which the total NaCl has been regarded as equal to the chloride in the serum plus NaCl added. The calculated increment in the freezing point measurement not due to chloride was  $0.171^\circ$ , the freezing point of the serum being  $0.532^\circ$ . These experiments are shown to agree with those made on serum with added sucrose and with added urea and would appear to indicate that NaCl was dissolved in the total water of the serum.

*Freezing Point of Serum Obtained from Febrile Dog and of the Same Serum with Sucrose Added*

From studies of fever produced by the intravenous injection of sugar and salt solutions Balcar, Sansum, and Woodyatt (10) advanced a theory that a free and bound water equilibrium existed normally in the body tissues and that during fever there was a greater binding power of the body colloids, causing a subsequent deficit of free water. In order to test the theory which Balcar, Sansum, and Woodyatt have formulated in reference to fever in so far as it concerns bound and free water in the sense in which we are using these terms, we made freezing point measurements on

TABLE II  
*Freezing Point of Ox Serum with Added NaCl*

Solution	Total NaCl	$\Delta t_{\text{obs.}}$	$\Delta t_{\text{NaCl}}$	$\Delta t_{\text{calc.}}$	$\frac{\Delta t_{\text{obs.}}}{\Delta t_{\text{calc.}}}$
	<i>N. per kg. H<sub>2</sub>O</i>	<i>°C.</i>	<i>°C.</i>	<i>°C.</i>	
Ox serum*	0.1027	0.532	0.361		
Same + NaCl	0.1559	0.716	0.542	0.713	1.005
	0.1982	0.860	0.686	0.857	1.004
	0.3042	1.212	1.041	1.212	1.000
	0.4057	1.554	1.380	1.551	1.002

\* The specific gravity of the serum was 1.0289  $\left(\frac{20^\circ}{20^\circ}\right)$ ; the total water content was 912.65 gm. per kilo of serum; the chloride concentration was 96.4 milli-equivalents per liter of serum.

serum obtained from a febrile animal similar to those made on normal serum.

A healthy dog was given an intramuscular injection of 10 cc. of milk. 6 hours after the injection the animal had a fever of 40.5° (rectal) and was bled by cardiac puncture. Serum was obtained from the defibrinated blood. The results of our measurements are shown in Table I. It will be seen that the ratio of the observed to the calculated freezing points is approximately unity. From this it would appear that in the case of serum from a febrile animal there was no change in the osmotic behavior of the water of that serum within the limits of error of our measurements.

*Effect of Added Urea on Freezing Point Depression of NaCl, Blood Serum, and Sucrose*

Hill has observed that certain substances such as urea and creatinine fail to exert their full effect on the vapor pressure of blood and corpuscles; this he attributed to a "slight degree of adsorption on, or combination with, the colloidal constituents or lipins of the blood." Grollman (11) also found that while urea produced the same depression of vapor pressure in solutions such as 1 per cent NaCl, Ringer's solution, and 1 per cent gelatin as it does when it is added in the same concentration in water, nevertheless, anomalous results were obtained when urea was added to

$$\frac{\Delta t}{N_w}_{1.04}$$

$N_w$ -Urea

FIG. 1. Molecular freezing point depression of urea in water

1.0  $N_w$  solution of sucrose. It thus seemed desirable to determine whether any change in the osmotic behavior of the water in blood serum occurred when urea was added.

In Fig. 1 is given our curve for the molecular freezing point depression of urea solutions in water. The only other molecular freezing point data of urea solutions which we have found aside from observations given in the International Critical Tables (three of which were in dilute solutions below 0.04  $N_w$ ) were made by Jones (12) with the usual Beckmann method. While Jones' measurements were made for the most part on more concentrated urea solutions (from 0.5 to 4.5  $N_w$ ), nevertheless he observed a similar decrease in the molecular freezing point lowering of urea solutions with increasing concentrations. This deviation from

the ideal is commonly observed with many other organic compounds. In the case of urea there is a suggestion that urea may

TABLE III

*Freezing Point Data of NaCl Solution, Ox Serum, and Sucrose Solution with Added Urea*

Solution	$\text{CO}(\text{NH}_2)_2$	$\Delta t_{\text{obs.}}$	$\Delta t_{\text{CO}(\text{NH}_2)_2}^*$	$\Delta t_{\text{calc.}}$	$\frac{\Delta t_{\text{obs.}}}{\Delta t_{\text{calc.}}}$
	$N_w$ per kg. $\text{H}_2\text{O}$	$^{\circ}\text{C.}$	$^{\circ}\text{C.}$	$^{\circ}\text{C.}$	
0.1605 $N_w$ NaCl		0.558			
Same + urea	0.0745	0.690	0.136	0.694	0.995
	0.0897	0.719	0.166	0.724	0.993
	0.1036	0.749	0.191	0.749	1.000
	0.2145	0.943	0.393	0.951	0.991
	0.2949	1.089	0.538	1.096	0.994
	0.4994	1.439	0.902	1.460	0.986
	0.5015	1.442	0.907	1.465	0.985
	0.8080	1.971	1.438	1.996	0.986
Average.....					0.991
Ox serum†		0.546			
Same + urea	0.0475	0.634	0.088	0.634	1.000
	0.0496	0.640	0.092	0.638	1.003
	0.0940	0.719	0.174	0.720	0.999
	0.2344	0.969	0.430	0.976	0.993
	0.2382	0.973	0.437	0.983	0.990
	0.4924	1.404	0.888	1.434	0.978
	0.5060	1.432	0.913	1.459	0.981
Average.....					0.992
Sucrose (0.275 $N_w$ )		0.526			
" + urea	0.0517	0.621	0.096	0.622	0.999
	0.1008	0.708	0.186	0.712	0.994
	0.2090	0.893	0.384	0.910	0.982
	0.3178	1.080	0.580	1.106	0.975
	0.5405	1.456	0.976	1.502	0.969

\* Calculated from the equation,  $\lambda = 1.858 - 0.1 (N_w)_{\text{urea}}$ .

† The specific gravity of the ox serum was 1.0270  $\left(\frac{20^{\circ}}{20^{\circ}}\right)$ ; the total water content was 914.51 gm. per kilo of serum.

form complex molecules, such as  $(\text{CO}(\text{NH}_2)_2)_2$  (13). Furthermore, our lack of information concerning equilibrium conditions between



urea and  $\text{NH}_4\text{CNO}$  and similar rearrangements permits us to consider the hypothesis of some type of association.

By plotting the molecular freezing point depression of urea against the molality, a straight line relationship may be obtained which may be expressed by the equation

$$\lambda = 1.858 - 0.1 (N_w)_{\text{urea}}$$

In Table III are given our results of the freezing point measurements made when urea was added to an NaCl solution, to oxserum, and to a sucrose solution. The average ratios of the observed to the calculated freezing point depression in the NaCl solutions and the ox serum were practically the same, being a trifle less than unity. The anomalous behavior of urea, however, is disclosed when the ratios of the observed to the calculated freezing point depressions of sucrose solutions to which urea had been added are considered. The results are in the opposite direction to those which might be expected if the effect were due to the hydration of sucrose. They lead to the suggestions that either a change in the activity of the solvent occurs or there is a chemical interaction between the sucrose and urea, or perhaps a change in the molecular structure of urea in the presence of sucrose.

#### DISCUSSION

Because of the lack of agreement between the theoretical and observed value of the osmotic pressures of sucrose (as shown by the freezing point data of Jones and Getman (14) and vapor pressure measurements of Scatchard (15)) sucrose has been regarded as being hydrated in solution. Newton and Gortner in their studies of the state of water in plant saps applied a correction for this hydration. Moran and Smith (16) and Grollman have recently criticized the method of calculation which Newton and Gortner employed, claiming that it did not take into account that the addition of sucrose to the original solution, if it is assumed to become hydrated, would increase the fraction of the final freezing point depression due to the solutes of the original solution as well as the fraction of the freezing point depression due to the sucrose added. Grollman's criticism, however, would appear to be invalid on the basis of our measurement of the freezing point depressions of a mixed solution of NaCl and sucrose. If it be

assumed that the sucrose is hydrated in solution, then it must also be assumed that in the case of NaCl and sucrose mixtures, NaCl is free to dissolve in the water of hydration of the sucrose since our observed freezing point depressions agree with the theoretical. A similar conclusion was arrived at by Corran and Lewis (17) who found by means of E.M.F. measurements on KCl-sucrose solutions that the observed activities for the Cl ion agreed with the theoretical only when calculated on the total water basis.

By means of vapor pressure measurements on NaCl-sucrose and KCl-sucrose solutions Grollman found that with  $0.75 N_w$  NaCl and  $0.093 N_w$  KCl respectively in  $1 N_w$  sucrose solutions the ratios of the observed to the theoretical vapor pressure lowering were unity, whereas in  $0.07 N_w$  NaCl and  $0.05 N_w$  KCl respectively in  $1 N_w$  sucrose the ratios were increased. Grollman believes that his result with the concentrated NaCl-sucrose solution "would indicate either a complete absence of any water of hydration or the dissociation of part of such water from the sucrose hydrate" and that his result with the less concentrated NaCl-sucrose solution "is compatible with the usually accepted view that sucrose is hydrated with the formation of a hexahydrate." Our freezing point measurements with  $0.0533 N_w$  NaCl-sucrose solutions do not confirm Grollman's vapor pressure measurements with his less concentrated NaCl-sucrose solution. Our results indicate that NaCl in the concentrations studied is dissolved in the total water present in NaCl-sucrose solutions, behaving as if no water of hydration of sucrose were present.

Objections have been raised to the application of freezing point data to biological fluids at physiological temperatures. It is conceded that vapor pressure measurements made at body temperature meet this objection. However, freezing point measurements appear to be in accord with vapor pressure measurements of blood, for Grollman (18) has shown that in the case of dog blood and plasma the vapor pressures calculated from experimentally determined freezing point data agreed with the values obtained from direct vapor pressure measurements made at  $37.5^\circ$ . Furthermore, Hill has shown by the use of his vapor pressure method that within the error of his method there is no change in the osmotic behavior of the water in blood when substances have been dissolved in blood. This is in accord with our present studies. The

criticism that the usual method for measuring the freezing point is liable to great sources of error, we believe has been obviated by our method.

While our freezing point studies indicate that within the error of our method all of the water of serum is "osmotically active" water, it is obvious that the data will not permit the calculation of the amounts of water involved in the hydration of the ions. The observed abnormalities in the freezing point depressions of concentrated solutions of electrolytes led Jones originally to the hypothesis of hydrate formation with ions. Evidence of the existence of hydrates has also been obtained from transport experiments in which the change in the concentrations at the electrode of an indifferent substance, such as raffinose, has been studied in a solution containing the indifferent substance and an electrolyte. Recently this hypothesis has been questioned (19) and there appears to be doubt as to whether ions are actually hydrated. Even assuming that hydration of ions exists, no significant change would be produced by this factor in our experiments with serum, since the concentration of the electrolytes in serum is low.

#### SUMMARY

The osmotic behavior of the water in blood serum was studied by means of freezing point measurements.

The calculated depressions of the freezing point of NaCl solutions and blood sera to which sucrose had been added were equal (within less than  $\pm 1$  per cent) to the observed depressions.

The calculated depressions of the freezing point of blood serum to which NaCl had been added were equal (within  $\pm 0.5$  per cent) to the observed depressions.

The calculated depressions of the freezing point of NaCl solutions, blood serum, and sucrose solutions, to which urea had been added were generally greater than the observed.

On the basis of our freezing point measurements it would appear that the total water of blood serum is capable of dissolving added solutes (sucrose, NaCl, or urea) within the range of concentrations studied, and that the total water of serum is "free" in the sense in which the term "free water" is used by Gortner and by Hill. Within the limits of error of our methods there would appear to

be no change in the osmotic behavior of the total water of serum taken from a febrile animal.

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# OXIDATION OF CYSTINE BY IODINE IN AQUEOUS MEDIUM

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## INTRODUCTION

The discovery and isolation of glutathione by Hopkins (1) opened up a new field of biochemical investigation in which the properties and functions of the sulfhydryl group ( $-SH$ ) are the major interest. At the present time research is being directed along three separate but associated lines. There are the metabolic studies of Lewis (2) and collaborators; there are the studies of Hammett (3) and collaborators, on the effect of the group and its partially oxidized derivatives on cell proliferation; and there are the studies of the oxidation mechanism by Dixon and Quastel (4), Michaelis and collaborators (5), Kendall and collaborators (6), and others (7).

The results of all these investigations indicate that a knowledge of the oxidation mechanism of the sulfhydryl group to its final stage is essential to an understanding of its biological functioning.

With this purpose the author started a year ago to find a reliable method to determine the sulfur compounds in various oxidation stages. The iodine (or iodate) method which was in common use was the first subject of investigation. The method was suggested by Rosenheim and Davidsohn (8) and was employed by Klason and Carlson (9). It was later modified by Okuda (10) to make it suitable for the determination of cysteine and cystine. This so called Okuda method has been extensively used by various workers with modifications or criticisms (11-19).

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As some of the workers have pointed out, the consumption of iodine (or iodate), from which the amount of cysteine or cystine is calculated, varies with the concentration of acid and of the sulfhydryl compound. Temperature of the solution to be titrated, speed of titration, and amount of shaking also greatly influence the iodine (or iodate) consumption. Moreover, the nature of indicators employed in acid titration influences the determination of the acid concentration, which in turn affects the iodine (or iodate) consumption, especially when the solution contains a large amount of zinc chloride.

Detailed discussion of each of these factors is omitted here except the effect of acid concentration, which is of primary importance in later developments.

*Effect of HCl Concentration on Consumption of Iodine (or Iodate)*

The variation in iodate consumption for solutions of the same cystine concentration but different HCl concentrations was studied. Okuda's procedure was strictly followed, in addition to the precautions which the author had found necessary in preliminary investigations. For instance, methyl orange was employed as an indicator in the titration of free HCl, thus excluding the acid combined with zinc; the temperature of the room was regulated to  $25^{\circ} \pm 1.0^{\circ}$  and the amount of shaking was arbitrarily fixed. The results are shown in Table I.

It is clear from Table I that the values deviate greatly owing to the very vague end reaction. No reliable figures can be obtained in such circumstances. However, Table I roughly indicates that the consumption of iodate solution greatly depends upon the acid concentration, and further, the change in cysteine concentration influences the consumption of the iodate solution.

One important conclusion from the above results is that the consumption of iodate solution exceeds that theoretically required for the oxidation of cysteine to cystine. Iodine produced by the interaction of  $\text{KIO}_3$  and HCl disappears rapidly until it reaches a certain point, after which the rate of disappearance becomes very slow; the higher the acid concentration, the slower the disappearance.

The theoretical consideration of this phenomenon was neglected even by workers in this particular field, except Bierich and Kalle

(14) who paid close attention to the overconsumption of iodine and proposed the following hypothetical explanation

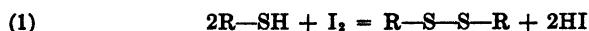


TABLE I

*Effect of HCl Concentration on Consumption of m/300 KIO<sub>3</sub> Solution*

HCl		KIO <sub>3</sub> consumed		HCl		KIO <sub>3</sub> consumed	
per cent		cc.		per cent		cc.	
0							
0.10		6.2		0.07		7.0	
0.27		5.5		0.24		5.8	
0.48		5.3		0.40		5.2	
0.65		5.2		0.58		5.05	
0.99		5.0		0.94		4.95	
1.92		4.8		1.32		4.85	
2.70		4.6		1.73		4.60	
3.12		4.5		3.50		4.25	
3.56		4.5					
4.45		4.3					
6.43		3.8					

Solution B. Cystine concentration  $2.0 \times 10^{-4}$  mols in 20 cc. Theoretical consumption of m/300 KIO<sub>3</sub> 20.0 cc.

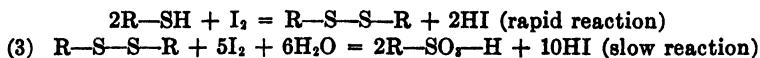
0.06	25.5-26.3	
0.10	26.9-27.9	
0.20	26.4-27.2	
0.47	25.4	
0.66	25.2	
0.90	24.7-25.0	
1.07	24.9-25.2	
1.35	23.7	
1.82	22.8	
2.10	23.0	
2.30	22.2-22.4	
3.02	21.8	

Nevertheless, they entirely neglected the possibility of oxidation of cystine by iodine, as did other workers likewise.

It occurred to the author that cysteine first might be rapidly



oxidized to cystine, or at least to an intermediate compound from which cystine results; secondly, cystine or the intermediate compound might be oxidized by excess iodine, as by bromine, to cysteic acid, in accordance with the following stoichiometrical equations.



where R represents  $-\text{CH}_2-\text{CH}(\text{NH}_2)-\text{COOH}$ .

To test this possibility a series of experiments was carried out.

#### EXPERIMENTAL

##### *Consumption of Iodine in Cystine Solution with Respect to Time*

*Materials. Cystine Solution*—A weighed amount of pure cystine<sup>1</sup> is dissolved in a definite amount of HCl solution of known concentration and diluted with water to a definite volume.

*Iodine Solution*—Baker's iodine (c.p.) after being dried in a desiccator was dissolved in water containing a definite amount of KI. Its normality was determined by 0.01 N  $\text{Na}_2\text{S}_2\text{O}_3$  solution before it was used.

*0.01 N Sodium Thiosulfate Solution*—Baker's sodium thiosulfate (c.p.) was dissolved in water through which purified nitrogen had been bubbled overnight. The solution was standardized against resublimed iodine.

*Technique*—Definite volumes of the cystine and iodine solutions which had been kept at  $25^\circ \pm 0.02^\circ$  were put in a 500 cc. volumetric flask and the total volume was made up exactly to 500 cc. by the addition of redistilled water at the same temperature. The reaction mixture was kept at  $25^\circ \pm 0.02^\circ$  in a water bath. At fixed intervals of time 25 cc. of the mixture were pipetted into a flask containing a little less of the standard sodium thiosulfate than is required. The slight excess of iodine in the solution was then titrated with the thiosulfate solution remaining in the same burette, starch being used as an indicator.

<sup>1</sup> Cystine used in the experiments was obtained through the courtesy of Dr. Gerrit Toennies of the Department of Chemistry of this Institute. The cystine is of the highest possible purity. Cf. (20).

From the total amount of the 0.01  $N$   $Na_2S_2O_3$  solution used the mols of iodine consumed were calculated.

All through the experiments the necessary directions reported by Rice, Kilpatrick, and Lemkin (21) were followed. However, the buffering of the reaction mixture to a neutral point before addition of the  $Na_2S_2O_3$  solution was omitted, because it had been observed that cystine reacts more rapidly with iodine in a solution of low acidity than in a higher one, giving greater errors than are caused by decomposition of  $Na_2S_2O_3$  in acid solutions. In fact, the amount of  $Na_2S_2O_3$  solution consumed in the titration of excessive iodine was observed not to vary perceptibly within the range of HCl concentrations employed in the author's experiments, as is shown in Table II.

TABLE II

*Consumption of 0.01  $N$   $Na_2S_2O_3$  Solution with Change in Concentration of HCl*  
Temperature  $25^\circ \pm 1.0^\circ$ .

Concentration of HCl	$Na_2S_2O_3$ solution consumed
<i>mol</i>	<i>cc.</i>
0	13.65
0.105	13.60
0.500	13.65
0.50	13.65
0.950	13.60

*Kinetic Experiment*—Since, owing to the slowness of reaction, the experiments extend over more than a week, it was suspected that the decrease in concentration of iodine is at least partly due to volatilization. Moreover, iodine may attack some other group of the cystine molecule besides the disulfide group. The former may be detected by the use of blanks and the latter by the use of *dl*-alanine. Experiments were therefore performed with the three solutions. Their constituents and experimental results are shown in Table III.

Results with Solution A and Solution B clearly indicate that there is no reaction between iodine and their constituents, the very slow disappearance of iodine from their reaction mixtures being no doubt due to losses by volatilization.

The results on Solutions B<sub>1</sub> and B<sub>2</sub> of Table III are plotted in

Fig. 1. This shows steady reaction velocity curves suggesting a common asymptote within experimental errors, which are prin-

TABLE III  
Rate of Disappearance of Iodine

Solution A (blank). HCl, $1.182 \times 10^{-4}$ mols; $I_2$ , $20.803 \times 10^{-4}$ mols; KI, 2.00 gm.					
Time		0.01 N $Na_2S_2O_3$ required per 25 cc. mixture			
hrs.		cc.			
0		10.40			
24		10.40			
48		10.40			
72		10.35			
96		10.30			
120		10.30			
336		10.20			

Solution C <i>d,l</i> -Alanine, $5.00 \times 10^{-4}$ mole HCl, $1.182 \times 10^{-4}$ mols $I_2$ , $20.300 \times 10^{-4}$ mols KI, 2.0 gm.		Solution B <sub>1</sub> Cystine, $2.502 \times 10^{-4}$ mols HCl, $1.182 \times 10^{-4}$ mols $I_2$ , $20.803 \times 10^{-4}$ mols KI, 2.00 gm.		Solution B <sub>2</sub> Cystine, $2.502 \times 10^{-4}$ mols HCl, $1.182 \times 10^{-4}$ mols $I_2$ , $17.697 \times 10^{-4}$ mols KI, 2.00 gm.	
Time	$I_2$ consumed*	Time	$I_2$ consumed*	Time	$I_2$ consumed*
hrs.	mols $\times 10^{-4}$	hrs.	mols $\times 10^{-4}$	hrs.	mols $\times 10^{-4}$
0	0	0	0	0	0
1.01	0	0.15	0.29	0.45	0.32
2.01	0.05	0.53	0.48	2.80	0.90
6.43	0.05	1.67	0.84	25.00	5.49
24.43	0.00	2.25	1.03	45.00	7.74
44.27	0.05	16.83	4.76	67.20	9.20
54.00	0.08	40.00	8.20	74.50	9.50
69.00	0.18	44.50	8.75	119.50	10.86
93.50	0.30	63.50	10.00	167.00	11.74
144.00	0.50	69.50	10.25	196.00	12.03
		112.00	11.92	214.30	12.23
		136.00	12.31		
		160.00	12.52		

\* Per 1000 cc. of solution.

cipally due to evaporation of iodine caused by the enlarging air space in the reaction bottles as the experiments proceed.

The amount of iodine necessary to oxidize  $2.50 \times 10^{-4}$  mols of cystine to cysteic acid is  $12.50 \times 10^{-4}$  mols by Equation 3.

The asymptote of the two curves is seen to correspond practically to this value, even without our taking the trouble for computation by an extrapolation formula. This is at least an indirect proof that the Equation 3 is quantitative.

#### *Isolation and Identification of Cystic Acid from Reaction Mixture*

In order to simplify the experimental conditions the addition of HCl and KI was omitted. Thus 2.5 gm. of pure cystine and 17.395 gm. of iodine were put in a glass-stoppered bottle to which

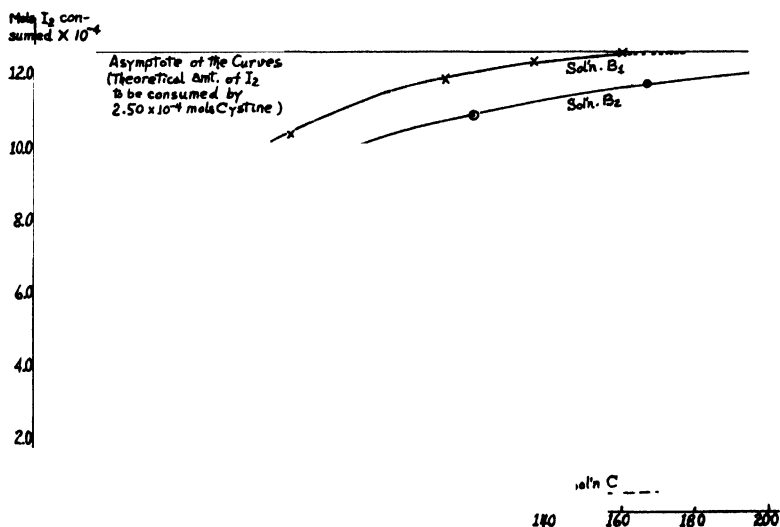


FIG. 1. The rate of disappearance of  $I_2$  in cystine solution

250 cc. of water were added. The stopper was then sealed with paraffin and the bottle was kept at room temperature ( $24-28^\circ$ ) with occasional shaking. At first the cystine and iodine remained almost undissolved and the aqueous layer showed only slightly brown coloration which was observed to increase very slowly with time. This is very likely due to the effect of HI produced by the reaction of cystine and iodine.

After 2 days all of the cystine was dissolved and only a small amount (roughly 0.5 gm.) of iodine remained undissolved, the aqueous layer being deep brown. At this point the reaction bottle

was warmed to about  $75^{\circ}$  in order to increase the reaction velocity and kept for 3 days at this temperature. The reaction mixture was then cooled to room temperature and diluted with water to 400 cc.

A titration of 5 cc. of the resulting solution showed that 13.159 gm. (0.0518 mol) of iodine had been consumed, which corresponds approximately to the amount of iodine theoretically required to oxidize the amount (0.0104 mol) of cystine used to cysteic acid according to Equation 3.

A titration of the solution used for the iodine determination by N NaOH solution indicated the presence of 0.135 N acid, which

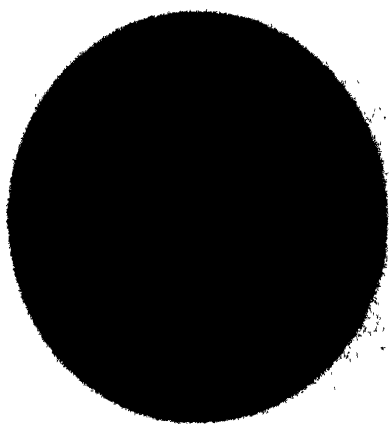


FIG. 2. Cysteic acid crystals;  $\times 30$

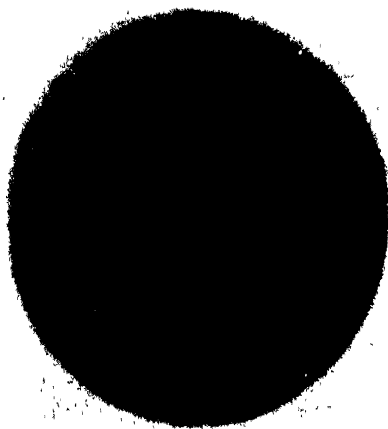


FIG. 3. Cysteic acid recrystallized;  $\times 45$

corresponds nearly to the total amount of acids (HI + cysteic acid) expected from the equation.

Hereupon the reaction mixture was transferred to a dish and carefully evaporated on a water bath at a temperature not exceeding  $60^{\circ}$ . On condensation of the solution, an almost colorless crystalline mass was found. After drying to remove as much hydriodic acid as possible, the mass was dissolved in dilute alcohol (about 40 per cent) and was recrystallized.

The yield of air-dried crystals was 3.0 gm. The crystal structure is shown in Fig. 2.

The mother liquid gave about 0.2 gm. of white crystalline sub-

stance showing a crystal structure under the microscope quite like the main portion.

The total yield was, therefore, 3.2 gm., which just corresponds to the amount of cysteic acid theoretically expected from 2.5 gm. of cystine.

TABLE IV  
*Physical Properties*

	Substance obtained by author	Cysteic acid obtained by Friedmann (22)	Inactive cysteic acid obtained by Gabriel (23)
	Colorless	Colorless	Colorless
Crystal form	Rhombic octahedron or prism of same system	Octagonal or prism	Octagonal system or polygonal column
Decomposition point	Darkening at 271°; foaming at 274°		Darkening and vivid foaming at 272-274°
Refractive index of crystal (24)	About 1.55		
Solubility	Very easily soluble in water; soluble in alcohol		
Specific refractivity	0.2045 (7.42 per cent solution used)		
Specific rotation	$[\alpha]_D^{25} = 8.246^\circ$ $[\alpha]_{Hg}^{25} = 10.308^\circ$	$[\alpha]_D = 8.66^\circ$	

The final mother liquid of dilute alcohol left a small amount of sticky amorphous substance on evaporation and gave a slight turbidity of BaSO<sub>4</sub> on the addition of BaCl<sub>2</sub> solution. This indicates that the reaction between cystine and iodine goes beyond the cysteic acid stage, possibly because of hydrolysis of cysteic acid. The amount of BaSO<sub>4</sub> precipitated was roughly less than 0.1 gm.

TABLE V  
*Chemical Properties*

	Substance obtained by author	Cysteic acid obtained by Friedmann (22)
Alkali fusion (25)	SO <sub>2</sub> , abundant production; H <sub>2</sub> S not detected	
Warming with alkali	NH <sub>3</sub> production	
Action of HNO <sub>2</sub>	N <sub>2</sub> gas production	
Amount of S	18.86 per cent (18.94 theory)	19.70 per cent 19.56 " " 19.00 " "
Amount of N (Kjeldahl)	8.20 per cent (8.28 theory)	8.44 per cent 8.82 " " 8.64 " "

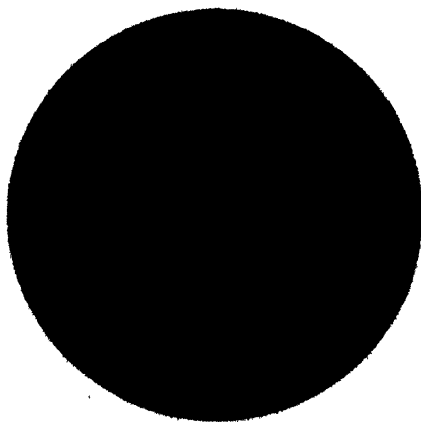


FIG. 4. Copper cysteinate crystals;  $\times 180$

and can, therefore, be neglected in the kinetic consideration if great accuracy is not required.

*Purification*—Having been washed with alcohol-ether mixture, 3.0 gm. of the oxidation product were dissolved in dilute alcohol

(about 40 per cent) from which the substance crystallized out in beautiful needles which are modifications of the octahedron (see Fig. 3).

The recrystallized crystals were dried in a desiccator over sulfuric acid, and lost 0.095 per cent of their weight on further drying at 110° under 25 mm. of pressure. The crystal form did not undergo any noticeable change by this treatment. The dried substance was analyzed to determine its identity and the results which are summarized in Tables IV and V are sufficient to identify the substance obtained by the author as cysteic acid.

*Copper Cysteinat*e—The cysteic acid solution was boiled with an excess of copper oxide for 5 hours and then filtered. The filtrate had the blue color of copper ions. When the filtrate was evaporated, a blue crystalline precipitate was obtained. The precipitate showed aggregates of minute hexagonal plates under the microscope, as is illustrated in Fig. 4.

#### *Analysis*

Cu 25.43 per cent. Calculated, Cu 25.57 per cent

The copper content is also in agreement with Friedmann's report (22).

#### DISCUSSION

After personal communication with the author, Dr. P. W. Preisler of the University of Pennsylvania conducted similar experiments with other disulfides and obtained their respective sulfonic acids. Therefore oxidation by iodine may be considered a general property of disulfides.

Discussion of the oxidation mechanism is omitted in this paper but will be reported on another occasion.

#### SUMMARY

1. Okuda's iodate method for cysteine and cystine determination was critically investigated, and it was shown that the iodine (or iodate) consumption in the method exceeds the theoretical amount required by the ordinarily assumed equation,  $2R-SH + I_2 = R-S-S-R + 2HI$ , as HCl concentration decreases.

2. It was definitely established that iodine, as do other oxidants,



oxidizes cystine to cysteic acid in an aqueous medium, in accordance with the stoichiometrical equation,  $R-S-S-R + 5I_2 + 6H_2O = 2R-SO_3-H + 10HI$ .

3. It was observed that there is a small amount of  $H_2SO_4$  produced in the reaction mixture.

4. Cysteic acid was isolated with a theoretical yield from the reaction mixture, and its physical and chemical properties were determined.

The author wishes to express his gratitude to Mr. R. McNeil, of the McNeil Chemical Company, Philadelphia, who supported this research, and also to his colleagues in the Research Institute of the Lankenau Hospital who assisted him in many ways. The author appreciates the advice and helpful criticism of Dr. M. Kilpatrick, Jr., of the University of Pennsylvania.

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## THE EFFECT OF CYANIDE UPON THE CATALYTIC ACTION OF DYES ON CELLULAR OXYGEN CONSUMPTION

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In previous communications (1) concerning the catalytic effect of reversibly oxidized and reduced dyes upon cellular oxygen consumption, it was stated that this action was insensitive to the addition of KCN which, as is well known, inhibits normal respiration. As proof for this statement, experiments were related showing that small concentrations of KCN which inhibited completely the respiration of the cells used (erythrocytes, leucocytes, marine eggs) had no effect on the dye catalysis, while narcotics of the type of urethane did produce an inhibition. It was furthermore stated that the dye catalysis was independent of the function of the respiration enzymes. It was also shown in the case of erythrocytes and leucocytes that the increased oxygen consumption was due to the oxidation of lactic acid (2).

Warburg, Kubowitz, and Christian (3) and Wendel (4), from Schaffer's laboratory, confirmed our results and found that the oxidized lactic acid is changed into pyruvic acid. Warburg postulated that the dye acts as a catalyst for the oxidation of hemoglobin and that it is the methemoglobin thus formed which produces the carbohydrate oxidation. Warburg was able to show in this thorough communication that methemoglobin alone can produce such oxidation. From these experiments on erythrocytes Warburg concludes that in the case of cells deprived of hemoglobin, hemin would replace the methemoglobin. If such was the case it would be possible to inhibit, at least partially, the catalytic effect of dyes by increasing the concentration of KCN. To test the validity of this hypothesis we have studied the dye catalysis on cells deprived of hemoglobin, namely the eggs of marine animals:

*Nereis*, sea urchin, and starfish. These experiments were performed in Woods Hole, during the summer of 1931.

*Toluylene Blue Catalysis and Effect of Increased Concentrations of KCN*

*Marine Eggs*—Since the catalytic effect of methylene blue is only one example of the many reversible oxidation-reduction systems capable of increasing cellular oxidations, we have chosen

TABLE I

*Effect of Increasing Concentrations of KCN upon Catalytic Effect of Dyes on Cellular Oxygen Consumption*

Marine eggs. Temperature 24°. Dye used, toluylene blue, 1.253 mm per liter. All these figures represent the averages of twelve to twenty experiments. After cyanide addition cell respiration was completely inhibited.

Concentration of KCN	O <sub>2</sub> consumption per hr.					
	Unfertilized <i>Nereis</i> eggs		Fertilized sea urchin eggs		Unfertilized starfish eggs	
	Before KCN addition	After KCN and dye addition	Before KCN addition	After KCN and dye addition	Before KCN addition	After KCN and dye addition
<i>M</i>	<i>c.mm.</i>	<i>c.mm.</i>	<i>c.mm.</i>	<i>c.mm.</i>	<i>c.mm.</i>	<i>c.mm.</i>
0 001	26 4	105 1	15 3	29 2	18.5	35.1
0 002	30 4	127 4	13.0	25 3	19.6	36.1
0 004	28 0	105 6	13 6	31 9	21 0	41.4
0 01	29.9	112 9	15.8	34.0	23.1	49.5
	Before dye addition	After dye addition	Before dye addition	After dye addition	Before dye addition	After dye addition
Without cyanide	23 0	130 0	14 0	44 0	23.0	59.2

toluylene blue for the experiments to be related. The concentration of the dye has been uniform throughout the experiments, namely 1.253 mm per liter. The concentration of KCN (Kahlbaum, previously brought to pH 8.2, the pH of sea water) has been varied from 0.001 M to 0.1 M. The oxygen consumption was determined by means of the Barcroft manometers with Warburg vessels: (a) before addition of KCN, (b) after the addition of KCN, and (c) finally after dye addition. In all cases the in-

hibition produced by cyanide was complete. In Table I we therefore give only the oxygen consumption before cyanide addition, after dye addition, and after cyanide and dye addition. Although the catalytic power of toluylene blue is diminished after the addi-

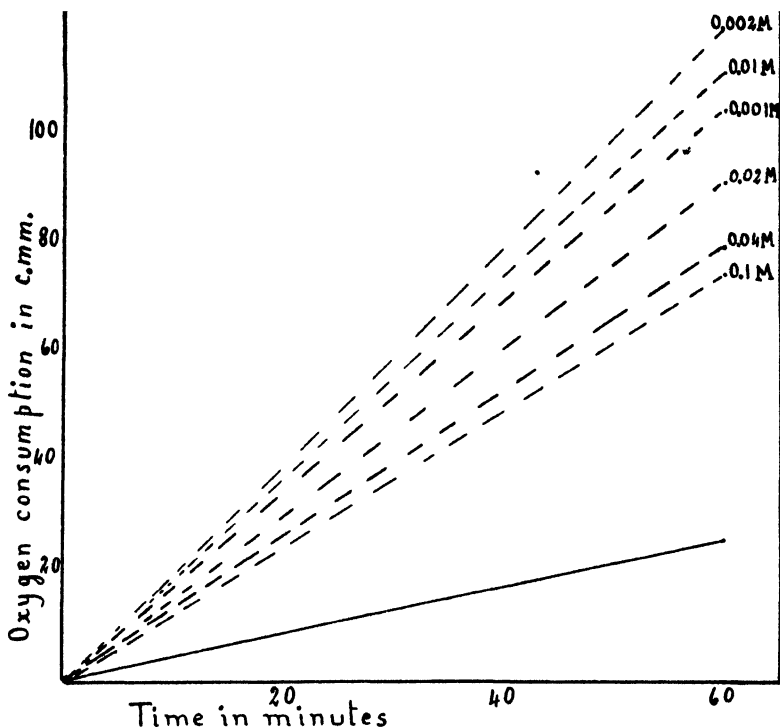


FIG. 1. The effect of toluylene blue on the respiration of *Nereis* eggs previously treated with increasing concentrations of KCN. The cyanide concentration was varied from 0.001 M to 0.1 M. The dye concentration was 1.253 mm per liter. The solid line indicates oxygen consumption of eggs before cyanide and dye addition; broken lines, oxygen consumption of eggs after the addition of toluylene blue, and increasing concentrations of cyanide. The numbers express the molar concentration of cyanide.

tion of cyanide, as shown when we compare the oxygen consumption of the cell after dye addition with the oxygen consumption after cyanide and dye addition, it can be seen in Table I that when the cyanide is increased 10-fold in concentration there is no

effect whatsoever on the catalytic action of the dye in the case of the eggs of *Nereis*, sea urchin, and starfish. The per cent increase of the oxygen consumption remains approximately the same, which would not be the case if this catalytic effect were due to the hemin present in the cell. Even when the cyanide is increased to such high concentrations as 0.1 M we have observed no inhibition of the dye catalysis. When the concentration is higher than 0.01 M cyanide there is a diminution of the catalytic power which is

TABLE II

*Effect of Increasing Concentrations of KCN upon Catalytic Effect of Dyes on Cellular Oxygen Consumption*

Dogfish erythrocytes. Dye, methylene blue, 5 mg. per cent. Temperature 24°. The dye has been added from the side arm of the vessels, the stop-cock of the manometers being kept closed.

Time	O <sub>2</sub> consumed (–) or produced (+) after KCN addition			
	0.1 M KCN	0.01 M KCN	0.001 M KCN	Control, no KCN
min.	c.mm.	c.mm.	c.mm.	c.mm.
30	–14.5	0	0	–16.6
Methylene blue added				
6	+13.6	+15.1	+2.5	–12.4
12	+19.3	+21.1	–0.9	–26.8
18	+21.1	+21.6	–8.1	–37.5
27	+19.0	+18.1	–21.0	–48.9
37	+16.4	+9.3	–33.0	–64.8
48	+15.3	+3.5	–41.5	–72.9
60	+7.3	–9.9	–51.6	–99.7
90	–10.2	–36.0	–70.9	–102.8

progressive as the concentration increases (Fig. 1) but this diminution is undoubtedly due to the increased cytolysis produced by cyanide at such high concentrations.

*Dogfish Erythrocytes*—Warburg, Kubowitz, and Christian have shown that increasing concentrations of KCN inhibit methylene blue catalysis on mammalian erythrocytes. We have observed a similar effect in experiments with dogfish erythrocytes. As the concentration of KCN is increased there is also an increased positive pressure due to the oxygen given out by the oxyhemoglobin.

The inhibitory action of KCN in the case of erythrocytes is probably due, as expressed by Warburg, partly to the strong affinity of cyanomethemoglobin and partly to cell cytolysis (Table II).

The effect of dyes on the oxygen consumption of mammalian erythrocytes is considerably enhanced by the addition of glucose (Michaelis and Salomon (5), Warburg *et al.* (3)). In the case of dogfish erythrocytes this effect is not so marked. Dogfish defibrinated blood was centrifuged, the serum removed, and replaced with isotonic NaCl (Kahlbaum). The suspension was buffered with phosphate buffer pH 7.4 (M/30 total concentration). The experiments were followed for 4 hours, and during this time the erythrocytes containing glucose as well as the ones without glucose showed

TABLE III

*Effect of Increasing Concentrations of KCN on Pyocyanine Catalysis of Cellular Oxygen Consumption*

Fertilized sea urchin eggs. Pyocyanine, 5 mg. per cent. Temperature 24°.

Concentration of KCN	O <sub>2</sub> consumption per hr.	
	Before KCN addition	After KCN and pyocyanine addition
<i>M</i>	<i>c.mm.</i>	<i>c.mm.</i>
0.001	16.04	26.2
0.002	17.7	22.4
0.01	17.9	24.7

a steady oxygen consumption, the increase due to the addition of glucose being only 19 per cent. The addition of glucose from 0.1 up to 2 per cent does not increase the normal respiration of these erythrocytes.

*Pyocyanine and Effect of KCN*—Friedheim (6) has reported another example of dye catalysis—pyocyanine, which increases the oxygen consumption of bacteria. We have previously stated (7) that all reversible oxidation-reduction dyes which penetrate into the cell and are capable of being reduced by it will act as catalysts provided the velocity of its reoxidation by atmospheric oxygen is not very low. We chose in those studies dyes with  $E_0'$  of different values and found that dyes from an  $E_0'$  of  $-0.160$  volt to  $+0.220$  at pH 7.0 act as good catalysts. The  $E_0'$  of



pyocyanine at such pH (Friedheim and Michaelis (8)) is  $-0.034$  volt. Friedheim's observation confirms once more the general character of this rule. Friedheim found that pyocyanine catalysis of bacterial oxidations is inhibited by cyanides. In the case of sea urchin eggs, increasing the concentration of KCN 10-fold has no effect on the catalytic action of pyocyanine (Table III).

#### DISCUSSION

Warburg and his coworkers explain the mechanism of the dye catalysis in red cells as follows: (1) methylene blue + hemoglobin +  $H_2O$  = methemoglobin + leucomethylene blue; (2) methemoglobin + carbohydrate = hemoglobin + oxidized carbohydrate; (3) leucomethylene blue +  $\frac{1}{2}O_2$  = methylene blue +  $H_2O$ . From these experiments on mammalian erythrocytes, they state that in cells deprived of hemoglobin hemin may take the place of hemoglobin. When erythrocytes are used as cell material, we believe there is present a double effect: (1) the catalytic action of the dye, (2) the oxidation produced by methemoglobin. The formation of methemoglobin through the addition of methylene blue could have been predicted on theoretical grounds by comparing the  $E_h$  of the oxidized dye and the  $E_h$  of the hemoglobin.

(The  $E_h$  of the system  $\frac{\text{methylene blue}}{\text{leucomethylene blue}}$  at a ratio 99:1 is  $+0.070$  volt at pH 7.0, while that of  $\frac{Hb}{MHb}$  at the same ratio is

$+0.050$  volt.) In the absence of oxygen, the oxidation of hemoglobin and the reduction of methylene blue would proceed until the establishment of a thermodynamic equilibrium, a condition which would be difficult to attain in the presence of oxygen. Conant and Fieser (9) have shown that the system Hb-MHb is a reversible oxidation-reduction system. Its  $E_o'$  at pH 7.4 is  $+0.090$  volt. The system falls, therefore, within the range of those reversible systems able to act as good catalysts for cellular oxidation (from  $E_o'$  of  $-0.160$  to  $+0.220$ ). The fact that methemoglobin does not act as a good catalyst, once reduced to hemoglobin by the oxidized carbohydrates, is due to the extreme sluggishness of the oxidizing action of atmospheric oxygen. In the case of methylene blue, the oxidation of the reduced dye is readily performed by molecular oxygen.

If the catalytic effect of reversible dyes on cells deprived of hemoglobin takes place through the oxidation of reduced hemin, the addition of increasing concentrations of cyanide would produce an inhibition of the dye catalysis, since, as Anson and Mirsky (10) have demonstrated, cyanide has a strong affinity for hemins. We have shown that increasing the concentration of KCN 10-fold does not decrease the catalytic power of the dye. Moreover the concentration of KCN can be increased to 0.1 M without inhibiting the catalytic power of the dye. We may, therefore, conclude that the catalytic power of reversible dyes upon the oxygen consumption of cells deprived of hemoglobin is due to a direct oxidation by the dye of the carbohydrate molecule rendered active and ready to be oxidized by the cellular enzymes.

#### CONCLUSION

The catalytic action of dyes upon the oxygen consumption of cells deprived of hemoglobin is not affected by the addition of increasing concentrations of KCN. This observation seems to prove the theory that the action of dyes is not related to a hemin catalysis but is a direct oxidation by the reduced dye of the carbohydrate molecule activated by cellular enzymes.

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# THE ANTIRACHITIC ACTIVATION OF ERGOSTEROL IN THE ABSENCE OF OXYGEN

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Most of the work on the antirachitic activation of ergosterol by exposing it to ultra-violet rays of a certain range has been carried out in the presence of oxygen. It is now established that prolonged irradiation of ergosterol in the presence of oxygen results eventually in the complete destruction, by oxidation, of the antirachitic substance which is first formed. However, what part, if any, oxygen plays in the activation of ergosterol has not been determined. Investigators have been divided in their views on this subject and absolutely conclusive experiments have not yet been done to elucidate this matter. Some investigators exposed ergosterol *in vacuo*, or in an atmosphere of nitrogen, in a quartz tube, to the action of ultra-violet rays of the proper range, and found that these procedures did not interfere with the antirachitic activation of ergosterol. The methods ordinarily used for the production of an atmosphere of nitrogen do not exclude the presence of an amount of oxygen greater than in the present study. All of the investigators who used a vacuum either failed to state the degree of evacuation which they succeeded in producing or failed to produce the high vacuum which can be obtained by modern technique for this purpose (1).

It has recently been found that oxygen in traces can affect reac-

tions (such as rates of polymerization) of certain organic compounds some of which have features in common with ergosterol; for example, citral (2) which has two double bonds. Since the facilities for the production of a very high vacuum were at hand, we considered it worth while to test the activation of ergosterol in an environment practically entirely free of oxygen.

It was considered that, in these circumstances, failure to activate the ergosterol by ultra-violet irradiation would either mean decomposition of this substance by the distillation prior to irradiation, or indicate that oxygen is absolutely necessary to bring about the activation. Successful activation under these conditions would mean that it can be effected in the virtual absence of oxygen.

We shall first give details of the method used for the purpose of irradiating the ergosterol in the absence of oxygen and then the details of the method and results of biological tests made on the irradiated products.

#### *Method of Distillation and Irradiation in Vacuo*

The ergosterol used for the experiments was obtained from Mead Johnson and Company, through the courtesy of Dr. C. E. Bills. It was distilled and irradiated under a total pressure which was less than  $10^{-4}$  mm., which represented the limit of the McLeod gage used for its measurement. The amount of oxygen present in the experimental tube was thus roughly one ten-millionth of that present at atmospheric pressure. Because the ergosterol was distilled in the vacuum before irradiation, thus freeing it from much of the oxygen which might be present as absorbed or occluded oxygen, or as "mloxides," the resulting distilled ergosterol probably contained relatively still less oxygen when compared with that ordinarily used in ergosterol studies. The vacuum obtained probably cannot be improved for the purpose at hand on account of impossibility of applying a "baking out" procedure in the presence of such an organic compound. The pertinent portion of the apparatus was, however, baked out *in vacuo* before introducing the ergosterol.

The apparatus used in preparing the sample was made entirely of Pyrex glass with the exception of the chamber in which the ergosterol was distilled and irradiated. This was of clear fused quartz and was attached to the vacuum system by means of a

graded glass seal. All tubing was of large size and stop-cocks of large bore in order to insure rapid evacuation and removal of evolved gases.

The vacuum was produced by means of a highly efficient two-stage mercury condensation pump backed by a Cenco oil pump, and was measured by means of a McLeod gage. The ergosterol was protected from mercury vapor, stop-cock grease, etc., by means of a liquid air trap, there being no stop-cock between this and the chamber containing the ergosterol.

The ergosterol to be distilled was placed in a small vessel which was lowered into the quartz chamber by means of a platinum wire and the system then made vacuum-tight by sealing off. The heating was accomplished by means of a heating element made of mica and nichrome wire which produced moderate uniformity in temperature but at the same time allowed the progress of the distillation to be observed. The element was not turned on until the highest vacuum had been reached. The distillation, which was in fact a sublimation, was carried out at 100–115°. Several hours were required for the appearance of a distinct white deposit of sublimate at this temperature. The pumps were kept running throughout the distillation and subsequent irradiation. At no time during the preparation was gas given off at a rate sufficient to be detectable on the McLeod gage.

The sublimate on the walls of the quartz tube was then irradiated at a distance of 30 cm. for a period of 30 minutes, a quartz mercury arc operating at 3.5 to 4 amperes at 220 volts being used. The irradiation was continued for a like period with the position of the lamp changed so that the direction of propagation of the rays made an angle of 90° with that of the first setting. This sample will be referred to as Preparation I.

After thoroughly cleaning the quartz vessel with chromic-sulfuric acids, a new sublimation *in vacuo* was carried out which differed from the former only in that the ergosterol to be distilled was contained in a special Pyrex tube so constructed that about half of the sublimate would escape and be deposited on the wall of the quartz and about half would be trapped in the upper portion of the tube which was carefully covered with tin-foil to protect it from the ultra-violet rays in the subsequent irradiation. This will be referred to as Preparation II of which there was an irradi-

ated and an unirradiated portion. The irradiation in this instance was carried out with the same lamp for 30 minutes at 30 cm. from one direction and 30 minutes at 30 cm. from an angle of 120° from the original direction of propagation of the rays.

In a third preparation the sublimation, carried out under the same conditions but from a simple glass test-tube, was continued until there was sufficient sublimate for a melting point determination. It melted sharply at 145° (uncorrected).

After irradiation, the ergosterol sublimate in Preparations I and II was dissolved in absolute ether. The concentration in the ether was determined by evaporation of an aliquot portion and weighing the residue. A measured amount of the ether solution was then dispersed in a measured amount of vitamin D-free olive oil, thoroughly mixed and warmed on the water bath until the oil was free of ether and the ergosterol remained completely dissolved in the oil. It was then kept at room temperature. The antirachitic potency of the two samples of irradiated ergosterol and of the sample of sublimated, but not irradiated, "ergosterol" was then determined by biological tests with the "prevention" method.

Rats about 4 weeks of age, most of them weighing between 45 and 55 gm., were used for the tests. A Roentgenogram of every rat was taken at the beginning of the test period. The animals were all fed on Diet 2965 of Steenbock and Black (3) and a Roentgenogram was taken at weekly intervals for 3 or 4 weeks, during which time various amounts of the different samples of irradiated and unirradiated ergosterol were given daily.

### Results

The results obtained at the end of the experimental period are given in Table I. It is seen that of each preparation of ergosterol that was first sublimated and then irradiated by a mercury vapor quartz lamp, in the *absence* of oxygen, about 0.00038 mg. was required daily as a protective antirachitic dose. The portion of sublimated Preparation II that was protected from irradiation was antirachitically inactive. Control rats that received no irradiated ergosterol developed moderate to severe rickets.

Ergosterol exposed to a mercury vapor quartz lamp in the *presence* of oxygen is known to acquire a potency that varies under different conditions of irradiation from 0.001 to 0.0001 mg. as the

TABLE I

*Effect of Ergosterol, Sublimed and Irradiated in Absence of Oxygen, upon Prevention of Rickets*

Rat No.	Daily dose of irradiated ergosterol	Condition of bones at end of experimental period (Roentgenogram)
Preparation I, sublimed and then irradiated <i>in vacuo</i>		
	mg.	
1	None	Moderate rickets
2	"	" "
3	0.00038	" "
4	0.00038	No rickets (complete protection)
5	0.00038	" " " "
6	0.00076	" " " "
7	0.00076	" " " "
8	0.00113	" " " "
9	0.00113	" " " "
10	0.00144	" " " "
11	0.00288	" " " "
12	0.00432	" " " "
13	0.00432	" " " "
Preparation II, sublimed and then irradiated <i>in vacuo</i>		
14	None	Severe rickets
15	"	" "
16	0.00048	No rickets (complete protection)
17	0.00038	" " " "
18	0.00038	" " " "
19	0.00038	Moderate rickets
20	0.00038	" "
21	0.00025	" "
22	0.00025	" "
23	0.00013	" "
24	0.00013	" "
25	0.00013	" "
26	0.00010	Severe "
27	0.00010	Moderate (healing) rickets
28	0.00008	Severe rickets
29	0.00002	" "
Preparation II, sublimed <i>in vacuo</i> but protected from irradiation		
30	0.026	Moderate rickets
31	0.0054	" "
32	0.00078	Severe "



minimal daily protective dose. 0.0002 mg. per day was the minimal protective dose of another sample of ergosterol obtained from the same source and irradiated at the same distance and for the same period in the presence of air. Therefore, the minimal protective dose of the preparations sublimated and then irradiated in the absence of oxygen falls within the range of values that have been reported for preparations irradiated in the presence of oxygen under various conditions.

#### SUMMARY AND CONCLUSIONS

In order to determine the part played by oxygen in the formation of vitamin D, *in vitro*, crystalline ergosterol, enclosed in a quartz tube, was first distilled in a high vacuum (less than  $1 \times 10^{-4}$  mm. of Hg) and then the sublimated ergosterol, practically in the absence of oxygen, was irradiated through the quartz tube by a mercury vapor quartz lamp. The irradiated product and a portion of the sublimated ergosterol that was protected from irradiation were then tested for antirachitic potency by the "prevention" method.

From the results obtained it is safe to conclude that distillation alone, in the absence of oxygen, does not activate ergosterol antirachitically and does not prevent its activation by exposure to a source of ultra-violet rays of the proper range. If oxygen plays any part in the antirachitic activation of ergosterol (the formation of vitamin D), it must be an amount less than that which was available under the conditions described. The antirachitic potency of the ergosterol irradiated in the absence of oxygen was within the range and no greater than that reported for preparations that were irradiated for an equal period in the presence of oxygen.

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# FACTORS INFLUENCING THE DISTRIBUTION AND CHARACTER OF ADIPOSE TISSUE IN THE RAT

## II. THE EFFECT OF OVARIECTOMY AND OF FEEDING WITH THYROXINE\*

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In a study of the influence of diet, undernutrition, fasting, and activity upon the distribution and character of adipose tissue in the rat, recently reported from this laboratory (Reed, Yamaguchi, Anderson, and Mendel, 1930), it was found that whereas the total amount of stored fat varied with the nature of the ration, the distribution was practically independent of the type of diet fed. Furthermore, it was discovered that, in general, none of the conditions imposed, except diet, altered the chemical nature of the deposited fat.

The present communication deals with a continued study of the factors influencing fat deposition. Data are presented to show the effect of complete ovariectomy and of feeding thyroxine, a substance known to increase the metabolic rate.

### *Experiments*

A description of the ration consisting of dried skimmed milk and coconut oil, which furnished 40 and 60 per cent respectively of the calorie intake in addition to supplements of yeast and cod liver oil, is published elsewhere (Reed, Yamaguchi, Anderson, and Mendel, 1930) together with an outline of the methods of dissection and extraction of the fatty tissue; also the character of the fat as indicated by the iodine number.

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Double oophorectomy was performed upon young rats previous to the onset of estrus, when they were 24 to 26 days of age. They were killed at ages varying from 78 to 124 days. Five of the group, consisting of seven animals, were over 100 days of age at the termination of the experiment (Table III). In members of the control (non-ovariectomized) lot of rats, the vaginal orifice opened at 60 to 70 days of age whereas in all ovariectomized individuals, it remained closed until the animals were killed. If oophorectomy is complete in rats spayed subsequent to the attainment of sexual maturity, the usual cyclic changes in the reproductive tract cannot be demonstrated in vaginal smears. We adopted for our criterion of complete ovariectomy the non-disappearance of the vaginal membrane inasmuch as Long and Evans (1922) reported that in 80 per cent of their 193 normal rats, the first estrus was either coincident with or following the establishment of the vaginal orifice by 5 to 10 days.

Other groups of female rats were fed a synthetic crystalline preparation of thyroxine mixed with the daily supplement of brewers' yeast. In one lot of rats the thyroxine feeding was initiated at 100 gm. of body weight with 0.5 mg. of thyroxine daily, the dose being increased gradually over a period of 60 days to 2 mg. daily. In another group the daily feeding of 2 mg. of thyroxine was deferred until the rats attained 200 gm. of body weight in order to determine whether a large dose given to an adult animal would produce a decline in body weight. On the assumption that the oral administration of thyroxine would at least prevent the usual increments in body weight, both groups of experimental rats were compared with control animals on an age basis, and furthermore as the average age at time of killing of each group of rats fed thyroxine differed by not more than 17 days (Table V) one lot of control animals was made to serve for both groups of experimental rats.

In order to demonstrate that the thyroxine was not being destroyed in the gut or otherwise rendered ineffective, metabolism measurements of two rats fed 2 mg. of thyroxine daily after the attainment of 200 gm. of body weight, 44 and 49 days respectively subsequent to the initiation of thyroxine administration, were made with a multiple chamber respiration apparatus described by Benedict (1930-31). The heat production was calculated from

the measured oxygen consumption which was considered to have a caloric value of 4.702 calories per liter, on the assumption that the fasting respiratory quotient is 0.72. The surface area was estimated by means of the formula,  $S = 9.1 \times W^{\frac{1}{2}}$  (Table I).

TABLE I\*  
*Heat Production of Female Albino Rats*

Metabolism at 30°, 24 hours after fasting.

Group of rats	Rat No.	Age	Weight	Rectal temperature at end of experiment	O <sub>2</sub> used in 6 hrs. per 200 gm. body weight	Heat production	
						Calories per sq. m. in 24 hrs.	Variation from average values of control groups of rats
		days	gm.	°F.	cc.		per cent
Thyroxine-fed	134†	109	193	102.4	2876	1717	+140.1
	136†	113	204	103.4	2849	1735	+142.7
Controls, without thyroxine	135	109	232	101.8	1110	704	715
	137	112	210	101.8	1184	726	
Ovariectomized	106	65	172	101.2	1415	814	-1.2
	107	65	154	99.8	1509	837	+1.6
	165	77	195	100.4	1387	831	+0.9
	181	62	175	100.4	1444	834	+1.2
Controls, non-ovariectomized	169	77	183	100.8	1435	843	824
	161	79	171	100.2	1274	732	
	179	62	166	100.8	1580	897	

\* The authors wish to acknowledge their indebtedness to Dr. Kathryn Horst of the Carnegie Institution of Washington, D. C., working at Yale University, for the determination of the data presented in this table.

† Measurements were made on Rats 134 and 136, 44 and 49 days respectively subsequent to the initiation of thyroxine feeding at 200 gm. of body weight. Only Rat 136 was active more than 20 per cent of the duration of the experiment.

Horst, Mendel, and Benedict (1930-31) have already discussed the relative merits of the different formulas proposed for estimating the surface area of the rat.

Another indication of the physiological activity of thyroxine and thyroid preparations, namely the hypertrophy of certain

internal organs, has been used extensively by Cameron and Carmichael (1921) to determine the activity of thyroid glands. The weights of the kidneys and livers of the animals listed in Table II show a similar marked degree of hypertrophy found in our group of rats fed thyroxine.

TABLE II  
*Hypertrophy of Internal Organs of Rats Fed Thyroxine*

Group of rats	Rat No.	Final body weight	Weight of liver	Weight of kidneys	Proportion of body weight	
					Liver	Kidneys
		gm.	gm.	gm.	per cent	per cent
Thyroxine feeding initiated at 100 gm. body weight	109	194	10.0	2.6	5.2	1.3
	110	188	11.3	2.6	6.0	1.4
	111	185	8.0	2.0	4.3	1.1
	112	183	10.0	1.5	5.5	0.8
Average.....		188	9.8	2.2	5.3	1.2
Thyroxine feeding at 200 gm. body weight	118	245	15.5	1.7	6.3	0.7
	126	252	11.2	2.2	4.5	0.9
	134	216	11.0	2.0	5.1	0.9
	136	229	14.5	2.3	6.3	1.0
Average.....		236	13.2	2.1	5.6	0.9
Controls, without thyroxine	108	179	7.5	1.0	4.2	0.6
	135	246	8.0	1.4	3.3	0.6
	137	223	8.0	1.4	3.6	0.7
	159	240	10.0	1.2	4.2	0.5
Average.....		222	8.4	1.3	3.8	0.6

#### RESULTS AND DISCUSSION

Reports are current that gonadectomy promotes fatness. Stotsenburg (1913), for example, observed that spayed rats showed more fat at autopsy than did non-spayed companion animals. Slonaker (1930) reported a greater increment in body weight of ovariectomized rats than of normal female rats, and suggested that the observation could be accounted for by increase in body length as well as in fatness. The final body weights of some of our oophorectomized animals (Table III) were well above some

individuals of the control group whereas the fat content of the two lots was practically the same. The ranges in the proportion of total depot fat to body weight in our spayed and control groups of rats were 11 to 17 per cent, and 8 to 19 per cent respectively; i.e., not significantly different. These data are considered to support indirectly the view that the increase in body weight fol-

TABLE III  
*Distribution of Fat in Ovariectomized Rats*

Group of rats	Rat No.	Age	Final body weight	Proportion of depot fat to total depot fat						Proportion of total depot fat to body weight
				Intermuscular	Genital	Subcutaneous	Perirenal	Mesenteric	Omental	
		days	gm.	per cent	per cent	per cent	per cent	per cent	per cent	per cent
Ovariectomized	160	128	226	9.7	16.6	52.7	12.7	6.3	2.0	15.8
	163	129	251	8.2	13.8	55.0	14.7	5.5	2.8	16.7
	165	89	213	6.5	13.3	56.9	12.3	7.4	3.6	11.2
	172	101	284	9.4	13.2	55.7	13.8	4.9	3.0	17.4
	173	102	266	9.1	9.5	63.5	11.0	4.8	2.1	11.9
	174	103	281	9.6	13.1	52.6	14.7	6.6	1.4	15.4
	181	77	240	8.0	10.9	55.8	11.9	10.9	2.7	10.9
Average.....		104	252	8.7	12.9	56.0	13.0	6.6	2.5	14.2
Controls, non-ovariectomized	161	128	232	7.5	24.7	37.9	19.4	7.6	2.9	11.4
	164	129	199	8.1	24.2	45.0	12.3	7.8	2.6	15.5
	169	91	198	8.0	24.1	45.4	14.3	5.7	2.5	9.5
	178	102	238	9.1	20.3	49.7	10.4	7.4	3.1	18.6
	179	77	213	11.2	18.5	43.7	11.0	10.9	4.7	8.2
Average.....		105	216	8.8	22.4	44.3	13.5	7.9	3.2	12.6

lowing ovariectomy may be due to greater skeletal growth rather than to notable increments in fatness. Cramer and Marshall (Marshall, 1922, pp. 390 and 391) report that they did not observe any marked tendency to deposition of fat in their castrated (female) rats. Apparently fattening follows gonadectomy more regularly in some species than in others. Korenchevsky (1925) remarks, "birds develop obesity so regularly after the removal of

the sexual glands that this operation is employed commercially for fattening animals for food."

Regarding the distribution of fat, however, it is interesting to note the marked decrease in the proportion of genital fat with a corresponding increase in the proportion of subcutaneous fat in the spayed rats (Table III). It seems plausible to attribute the decrease of genital fat to a disturbance or decrease in the blood supply to the genital organs as a result of the operative procedure. The fact that all the other depots did not receive an equal share

TABLE IV  
*Iodine Values of Subcutaneous Fat of Ovariectomized Rats*

Group of rats	Rat No.	Iodine No.
Ovariectomized	160	31.6
	163	27.7
	165	29.4
	172	33.5
	173	33.9
	174	32.3
	181	34.6
Average.....		32.0
Controls, non-ovariecto- mized	161	36.3
	164	34.4
	169	36.4
	178	34.3
	179	36.2
Average.....		35.5

of the fat that presumably would have gone to the genital region cannot be so easily explained.

The character of the fat as indicated by the iodine number of the subcutaneous fat of ovariectomized rats was similar to that of the control non-spayed animals (Table IV). In rats ovariectomized 40 to 50 days previously, striking metabolic changes as represented in heat production values were not revealed (Table I).

The proportionate distribution of fat in most depots of both groups of rats fed thyroxine was similar to that of the control animals which did not receive the hormone (Table V). On the

other hand the proportionate amounts of fat in the selected depots of the rats fed thyroxine were less than one-half that found for the control lot of animals.

The failure of the heavier rats (Group 3, Table VI) fed thyroxine to decline in body weight may be explained by their greatly

TABLE V  
*Distribution of Fat in Rats Fed Thyroxine*

Group of rats	Rat No.	Age	Final body weight	Proportion of total depot fat						Proportion of total depot fat to body weight
				Intermuscular	Genital	Subcutaneous	Perirenal	Mesenteric	Omental	
		days	gm.	per cent	per cent	per cent	per cent	per cent	per cent	per cent
Thyroxine feeding initiated at 100 gm. body weight	109	132	194	7.4	21.5	42.9	15.9	8.4	3.9	5.6
	110	102	188	5.6	29.4	40.7	15.4	6.9	1.8	6.4
	111	130	185	5.2	18.9	49.9	15.5	8.6	3.6	8.1
	112	127	183	8.2	23.1	41.2	17.3	7.0	3.2	5.2
Average.....		123	188	6.6	23.2	43.7	16.0	7.7	3.1	6.3
Thyroxine feeding at 200 gm. body weight	118	172	245	5.8	17.7	48.2	14.4	11.6	2.3	2.9
	126	152	252	10.3	25.5	35.4	16.4	8.5	3.9	4.8
	134	113	216	10.1	21.8	39.5	18.4	8.1	2.1	8.7
	136	124	229	9.3	21.2	45.5	14.5	7.0	2.5	9.0
Average.....		140	235	8.9	21.5	42.2	15.9	8.8	2.7	6.4
Controls, without thyroxine	108	105	179	5.5	22.3	49.7	13.4	6.6	2.5	8.3
	135	114	246	7.8	18.5	46.7	13.0	5.0	2.0	18.7
	137	126	223	9.7	24.3	39.4	18.9	5.3	2.4	12.4
	159	141	240	11.7	22.9	43.2	13.9	5.3	3.0	15.7
Average.....		122	222	8.7	22.0	44.9	14.8	5.6	2.5	13.8

increased food consumption. The average daily amount of food eaten by the thyroxine-fed animals of Group 3 was more than 12 gm. as compared with only about 7 gm. for members of Group 4 which were not fed thyroxine. Likewise the inability of the rats, which ingested thyroxine when they attained 100 gm. of body weight, to maintain the same weight as the controls, was presum-



ably due to the failure to increase their food intake adequately (Groups 1 and 2).

TABLE VI  
*Food Intake of Rats Fed Thyroxine*

Group of rats	Rat No.	Final body weight	Age at beginning of experimental period	Age when killed	Duration of experimental period	Food eaten during experimental period	Food eaten per day	Proportion of total depot fat to body weight
		<i>gm.</i>	<i>days</i>	<i>days</i>	<i>days</i>	<i>gm.</i>	<i>gm.</i>	<i>per cent</i>
Group 1; thyroxine feeding initiated at 100 gm. body weight	109	194	52	133	81	650		5.6
	110	188	52	103	51	380		6.4
	111	185	48	130	82	550		8.1
	112	183	52	127	75	550		5.2
Average.....		188	51	123	72	533	7.4	6.3
Group 2; controls (without thyroxine) to Group 1	108	179	51	105	54	330		8.3
	135*	246	39	114	75	570		18.7
	137*	223	39	126	87	550		12.4
	159*	240	45	141	96	880		15.7
Average.....		222	44	122	78	583	7.5	13.8
Group 3; thyroxine feeding at 200 gm. body weight	118	245	88	172	84	882		2.9
	126	252	90	152	62	780		4.8
	134	216	80	113	33	470		8.7
	136	236	85	124	39	610		9.0
Average.....		237	86	140	55	686	12.5	6.2
Group 4; controls (without thyroxine) to Group 3	135*	246	74	114	40	360		18.7
	137*	223	88	126	38	220		12.4
	159*	240	91	141	50	330		15.7
Average.....		236	84	127	43	303	7.1	15.6

\* At the beginning of their respective experimental periods, these rats of Group 2 weighed 100 gm. each, whereas these rats of Group 4 weighed 200 gm. each.

Absorption and utilization of the hormone were indicated in the increased heat production values, which were more than 140 per cent higher for rats fed thyroxine than the average value of control animals not fed thyroxine (Table I). The recorded data

represent rectal temperatures of the rats after they had sojourned at an environmental temperature of 30° for about 6 hours without access to water. It is not known whether the thyroxine-fed rats maintained the unusually high cell temperatures when they lived in a so called normal environment of approximately 24°.

All rats ingesting thyroxine stored fat that exhibits a higher iodine number than did their companion rats which did not receive

TABLE VII  
*Iodine Values of Depot Fat of Rats Fed Thyroxine*

Group of rats	Rat No.	Inter-muscular	Genital	Subcutaneous	Peri-renal	Mesenteric	Omental
Thyroxine feeding initiated at 100 gm. body weight	111	41.5	39.3	40.5	40.1	38.5	38.5
	112	44.1	42.3	44.4	41.5	41.2	42.4
Average.....		42.8	40.8	42.5	40.8	39.9	40.5
Thyroxine feeding at 200 gm. body weight	118	47.1	46.9	49.0	45.4	45.3	
	126	45.7	46.5	48.8	45.6	44.3	43.5
	134	45.2	46.2	45.7	43.6	38.3	42.6
	136	45.6	43.2	45.0	44.5	45.0	46.5
Average.....		45.9	45.7	47.1	44.8	43.2	44.2
Controls, without thyroxine	108	24.6	28.7	31.6	32.0	32.0	
	135	32.6	32.2	34.4	31.4	30.8	30.0
	137	29.6	35.9	37.2	35.7	33.7	33.8
	159	36.8	36.4	39.0	36.1	35.4	35.8
Average.....		30.9	33.3	35.6	33.8	33.0	33.2

The iodine value of the dietary fat, coconut oil, was 8.0.

the hormone. The animal organism alters certain dietary fats by a desaturating process. For example, coconut oil displays an iodine value of 8, whereas the body fat deposited in rats fed a ration rich in this food fat has an iodine number of about 33. A still more highly desaturated product was observed, however, in the fat of animals to which thyroxine was administered. All of the depot fats of the latter were found to yield higher iodine values—increments of 10 or 15 units—than the fats of the control

group which did not receive the drug (Table VII). This difference in saturation is noteworthy in that it seems to indicate some relationship between the degree of saturation of the stored fat and the metabolic rate of the animal.

Inasmuch as all of the trials reported herein were made with a diet rich in coconut oil, it remains to be determined whether in animals receiving a ration rich in other fats or in rats that are compelled to synthesize their fat from carbohydrate, the administration of thyroxine will likewise alter the character of body fat.

#### SUMMARY

In rats completely ovariectomized at an age of between 3 and 4 weeks and killed 7 to 14 weeks later, there was a negligible difference in the percentage content of fat in the body over that of intact animals. Ovariectomized rats stored a smaller proportion of fat in the genital tissues and a larger proportion in the subcutaneous layers than did their non-spayed companion animals. The quality (iodine number) of the subcutaneous fat was not altered by ovariectomy.

In one group of rats of 100 gm. of body weight, the daily dose of thyroxine was gradually increased from 0.5 mg. to 2 mg. Another lot of rats of 200 gm. of body weight received daily doses of 2 mg. of thyroxine from the beginning of the test. In both groups the distribution of fat in most of the depots was similar to that of the control rats that did not receive thyroxine. The percentage content of depot fat in the entire body of rats fed thyroxine was, however, less than one-half that found for the control animals. Furthermore, the depot fat produced by rats fed thyroxine was more unsaturated, as determined by higher iodine values—increments of 10 or 15 units—than the fat yielded by rats not fed thyroxine.

All results reported in this communication were obtained with rats fed a ration rich in coconut oil.

Of all the factors, including food, undernutrition, fasting, muscular activity, ovariectomy, and the administration of thyroxine, which we have studied thus far, the character of the diet and the thyroid hormone represent the only influences that have appreciably altered the quality of depot fat.

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## THE OXIDATION OF PROLINE AND OXYPROLINE BY LIVER

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It has been shown (1) that pyrrole acts as a catalyst in certain oxidations in liver. It was therefore interesting to test the action of proline and oxyproline to see whether they behaved analogously. The behavior of these amino acids is, however, quite different, for they themselves are definitely oxidized. This difference in behavior from pyrrole may be accounted for by the introduction of the COOH group, making the ring more vulnerable to attack, especially the hydrogen atom attached to the neighboring nitrogen.

There have been no reports on the biological oxidation of proline or oxyproline. Fürth and Kaunitz (2) showed that they were oxidized on a charcoal surface with the splitting of the ring and the liberation of ammonia. The oxidation by liver is not as drastic. The ring is probably not broken and no ammonia is given off. The end-product has not been identified and awaits a large scale experiment. A clue to the possible end-product can be obtained from the study of the oxygen uptake.

### *Technique*

The Barcroft-Warburg apparatus was used. Rat liver was chopped with scissors, weighed, and 1 cc. of buffer added for every gm. of liver. The mixture was ground in a mortar and squeezed through muslin so that an even suspension was obtained. The *l*-proline was prepared by Town's (3) method, and *l*-oxyproline by Klabunde's (4) method. Neither proline nor oxyproline when shaken in the apparatus alone or with boiled liver takes up any oxygen, so the oxidation is due to a thermolabile catalyst.

If 1 atom of oxygen were taken up for every molecule of proline, then theoretically every mg. of proline ought to take up 97

c.mm. of oxygen. As shown in Fig. 1, 1 mg. of proline takes up just half this amount; in other words, 2 molecules of proline share 1 atom of oxygen. The concentration of the enzyme has, however, some influence. The optimum for the oxidation is about pH 8.0, and with the standard preparation the uptake is from 60 to 70 c.mm. of oxygen in 60 minutes. At this pH there is a tendency for more than half the theoretical amount of oxygen to be taken up if the oxidation is proceeding rapidly. Dilution of

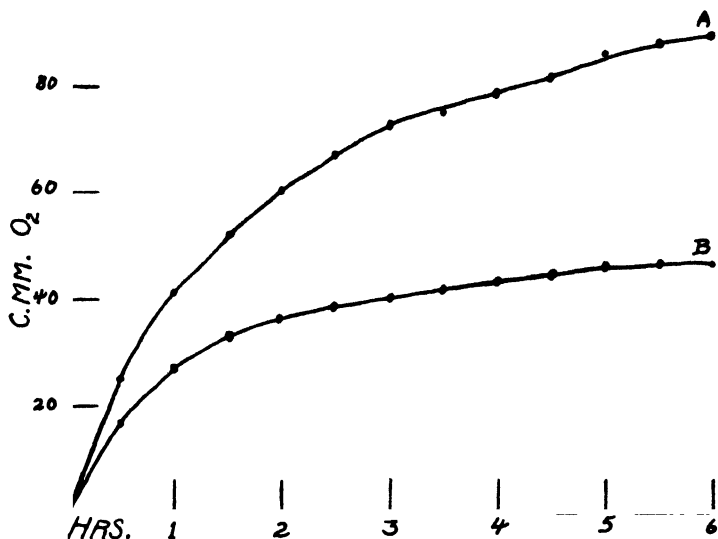


FIG. 1. Curve A represents the oxygen uptake of liver and 2 mg. of proline minus the uptake of liver alone; Curve B, the same with 1 mg. of proline. The theoretical uptake for 1 mg. of proline is 97 c.mm. of oxygen.

the enzyme at this pH slows the reaction, prevents this excess uptake, and gives theoretical values. Also at pH 7.0 or pH 9.0, where the oxidation is slower, exactly half the theoretical oxygen uptake occurs. This excess uptake at pH 8.0 must be due to the fact that the oxidation is so rapid that the condensation of 2 molecules of proline which must occur cannot take place rapidly enough, thus allowing for possible further oxidations. Fig. 1 shows the oxygen uptake at pH 7.8 with liver diluted twice.

This effect of pH is not a consideration in regard to oxypoline.

Although its oxidation has the same pH optimum as proline, yet the rate of oxygen uptake is much slower at the optimum as well as throughout the whole range. For every mg. of oxyproline 85.5 c.mm. of oxygen should be taken up. But here again the oxidation stops when half that amount has been used. This is shown in Fig. 2. The oxidation is very sensitive to the concentration of oxyproline. Thus 0.5 mg. will take up 21 c.mm. of oxygen, 1.0 mg. will take up 42 c.mm., but the oxidation of 2.0

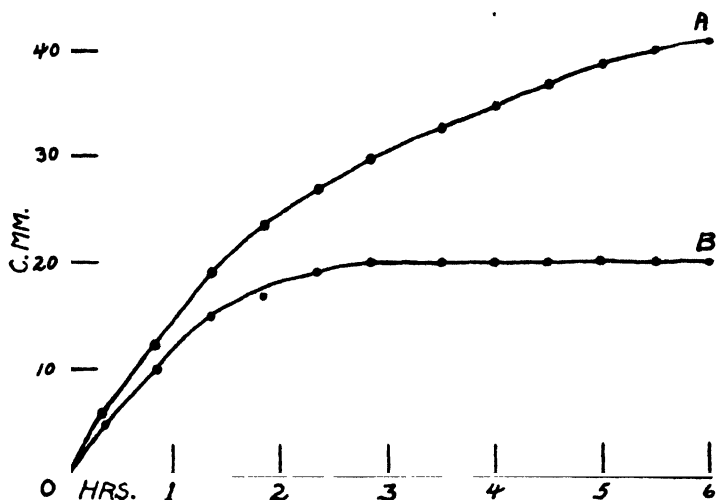


FIG. 2. Curve A represents the oxygen uptake of liver and 1 mg. of oxyproline minus the uptake of liver alone; Curve B, the same with 0.5 mg. of oxyproline. The theoretical uptake for 1 mg. of oxyproline is 85.5 c.mm. of oxygen.

mg. results in an uptake of only from 60 to 70 c.mm. Thus a concentration of oxyproline greater than 1 mg. tends to inhibit the oxidation. The proline oxidation is inhibited only when more than 3 mg. of proline are present.

This raises the question whether the oxidation of these two amino acids is due to the same catalytic system or to two distinct systems. In order to test this, the oxidation of proline and oxyproline together was compared with their oxidation separately. If two systems are involved, the oxygen uptake of the two sub-



stances together ought to be exactly equal to the sum of the separate oxidations during the whole course of the reaction. This,

TABLE I

*O<sub>2</sub> Uptake of Proline and Oxyproline*

pH 7.8; phosphate buffer containing 2 per cent sodium fluoride; 30°.

Time	O <sub>2</sub> uptake		
	2 mg. proline	2 mg. oxyproline	2 mg. proline 2 " oxyproline
	<i>c.mm.</i>	<i>c.mm.</i>	<i>c.mm.</i>
45 min.	46	11	49
1 hr., 45 min.	79	27	90
2 hrs., 40 "	95	36	109
4 " 5 "	112	48	129
5 " 15 "	119	51	139
6 " 45 "	124	53	148

TABLE II

*O<sub>2</sub> Uptake of 2 Mg. of Proline after 1 Hour at Various Hydrogen Ion Concentrations*

Phosphate and borate buffers containing 2 per cent sodium fluoride; 30°

pH.....	6.0	6.9	7.8	9.1
	<i>c.mm.</i>	<i>c.mm.</i>	<i>c.mm.</i>	<i>c.mm.</i>
O <sub>2</sub> uptake, after 1 hr.....	24	35	61	37

TABLE III

*Anaerobic Reduction of Methylene Blue by Proline and Oxyproline*

pH 7.8; phosphate buffer containing 2 per cent sodium fluoride; 18°.

Each tube contained 1 cc. of 1:5000 methylene blue and 0.25 cc. of liver suspension.

Tube No.	Buffer	Proline, 1 mg.	Oxyproline, 1 mg.	Reduction time
	<i>cc.</i>	<i>cc.</i>	<i>cc.</i>	<i>min.</i>
7	1.0			40
8	0.75	0.25		25
9	0.75		0.25	29

however, is not the case. The oxygen uptake of the two together is always less than the sum of their separate oxygen uptakes, indicating that one catalytic system is involved. This is shown

in Table I. Table II shows the effect of pH on the rate of oxidation.

KCN in a concentration of 0.005 M has no effect on the oxidation. 2 per cent sodium fluoride also has no effect. In fact, all the experiments were carried out in buffer containing 2 per cent sodium fluoride to prevent bacterial contamination. This is important, for there is evidence that bacteria can oxidize these amino acids further than liver. Washing the liver with relatively large quantities of water slows the rate of oxidation, indicating that the catalyst is partially soluble.

In order to determine whether any ammonia was given off during this reaction, the oxidation was allowed to proceed to completion at pH 6.9 to prevent ammonia from blowing off. The ammonia in the liver preparation alone and with the proline was then estimated according to the method of Hare (5). No difference was found, indicating that no deamination had taken place and that the ring was probably intact. There was also no decarboxylation.

As shown in Table III, the oxidation of both proline and oxyproline can take place anaerobically in the presence of methylene blue. This fact places the catalyst responsible in the class of dehydrogenases.

#### DISCUSSION

The fact that methylene blue is reduced and that only half the theoretical amount of oxygen is taken up by these amino acids suggests that 1 hydrogen atom is donated by each molecule of proline or oxyproline. The question is, in what part of the molecule does the oxidation occur? Pyrrole is not oxidized by the same liver preparation. Proline except for its saturation differs only from pyrrole in the introduction of a COOH group adjacent to the nitrogen. It is probable that the hydrogen attached to the nitrogen is involved in the oxidation and subsequent condensation. But there are many possibilities of rearrangement and the analysis of the mechanism awaits the isolation of the end-product.

#### SUMMARY

1. Both proline and oxyproline are oxidized by liver. On the basis of 1 atom of oxygen per molecule of amino acid one-half the theoretical amount of oxygen is taken up.

2. The oxidation has a pH optimum of about 8. KCN has no effect on the oxidation.

3. Methylene blue is reduced by both proline and oxypoline. The catalyst thus belongs to the class of dehydrogenases.

4. The end-product has not yet been identified.

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## THE ACTION OF COLLOIDAL SULFUR ON LIVER OXIDATIONS

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Colloidal sulfur has been used clinically for the production of fever in human beings, (see Shilvock (1) and Waller and Allen (2) for the literature). One animal experiment has also been reported. Piéry, Bonnamour, Milhaud, and Guiguonet (3) found that after injection of colloidal sulfur into rabbits,  $H_2S$  was evident in the breath and that 0.01 gm. per kilo was a toxic dose. Rastelli and Casazza (4) showed that incubation of colloidal sulfur with egg albumin or blood serum reduced the sulfur to  $H_2S$ . Abderhalden and Wertheimer (5) showed that colloidal sulfur accelerated the oxidation of aldehydes. It was therefore of interest to study its action on the oxygen uptake of tissue preparations.

For this purpose a liver preparation was used. It was soon obvious that the effects observed were due to the formation of  $H_2S$ . Liver incubated anaerobically with colloidal sulfur produced  $H_2S$  with great rapidity. Aerobically it was produced more slowly, but still in appreciable amounts. The experiments recorded below could be reproduced with  $H_2S$ .

The effect of  $H_2S$  on tissue oxidation has been studied by Negelein (6) who showed that the oxygen uptake of yeast was inhibited by small amounts, and by Keilin (7) who found that it inhibited the oxidation of cytochrome. Its inhibiting action on yeast fermentation was studied by Neuberg and Perlmann (8), and its action on the isolated heart by Koda (9). Krebs (10) showed that oxidations in which hematin compounds acted as the catalyst were inhibited by  $H_2S$ .

### *Rate of Production of $H_2S$ from Colloidal Sulfur*

A standard liver preparation was used throughout these experiments. Fresh guinea pig liver was finely minced with scissors,

weighed, and 1 cc. of buffer added for every gm. of liver. This mixture was ground in a mortar and squeezed through muslin, and an even suspension was thus obtained. Modified Thunberg tubes were used for the anaerobic experiments and the Barcroft-Warburg apparatus to measure oxygen uptake. The colloidal sulfur was prepared from sodium thiosulfate by the addition of sulfuric acid according to the method of Odén (11). After repeated precipitations, it was dialyzed for 3 days to get rid of the contaminating salts, and diluted so that 1 cc. contained between 0.3 and 0.4 mg. of colloidal sulfur. This solution was stable for several months. All experiments were carried out at 30° unless otherwise stated.

The first experiments were qualitative ones with lead acetate paper. 0.5 cc. of liver preparation, 1.3 cc. of phosphate buffer at pH 7.0, and 0.2 cc. of colloidal sulfur were placed in a Thunberg tube with lead acetate paper in the side arm. 15 minutes after evacuation, a distinct darkening of the paper was visible, which slowly deepened over a period of 2 hours. Liver without sulfur produced no darkening over a period of 6 hours. The same experiment was repeated aerobically, the lead acetate paper being put in the side arm of the Warburg manometer flask. The first darkening was seen after half an hour and deepened much more slowly than in the anaerobic experiment.

The effect of urethane was next tried. According to Keilin urethane inhibits the dehydrogenases and it inhibits the reduction of methylene blue by liver. It also inhibits the oxidation of fats (Loebel and Shorr (12)). It might therefore inhibit the reduction of colloidal sulfur. But on the contrary, it markedly accelerates its reduction. The above experiments were repeated; 0.7 cc. of a 20 per cent urethane solution in buffer replaced 0.7 cc. of the buffer alone, giving a final concentration of urethane of about 7 per cent. Both aerobically and anaerobically, darkening of the lead acetate paper occurred immediately. This effect is not due to the greater insolubility of  $H_2S$  in urethane solution. To test this point a given quantity of  $H_2S$  water was added to the buffer with and without urethane and these solutions shaken in the Warburg manometers. Samples were removed at intervals and titrated with iodine, and the amount of  $H_2S$  remaining in both solutions was found to be the same. All the above experiments were repeated at pH 6.0 with similar results.

This point was definitely proved by the following quantitative experiment. For this purpose methylene blue was used. The liver reduces the colloidal sulfur to  $H_2S$  and the  $H_2S$  then reduces the methylene blue. For this purpose a liver preparation previously washed with 100 cc. of distilled water and centrifuged was used to prolong the reduction time of methylene blue by the control. The results are given in Table I.

In spite of the fact that urethane inhibits the reduction of methylene blue by liver, it accelerates the reduction of colloidal sulfur. The acceleration is more marked than the difference between 75 and 55 minutes, for the control of the former was

TABLE I

*Reduction of Methylene Blue by Liver in Presence of Colloidal Sulfur and Urethane*

Each tube contained 1 cc. of 1:5000 methylene blue and 1 cc. of washed liver, except Tubes 6 and 7 which contained no liver.

Tube No.	Buffer, pH 7.0	Colloidal S	20 per cent urethane in buffer	Reduction time; 18°
	cc.	cc.	cc.	min.
6	1.5	0.2		$\infty$
7	1.0	0.2	0.5	$\infty$
8	0.7			125
9	0.5	0.2		75
10	0.2		0.5	160
11		0.2	0.5	55

reduced at 125 minutes, that of the latter in 160 minutes. These same time relationships hold at pH 6.0. Boiled liver does not show this effect, so it is dependent on some thermolabile system. Washed muscle reduces colloidal sulfur very slowly.

#### *Effect of Colloidal Sulfur on Oxygen Uptake*

In using the Barcroft-Warburg apparatus for this work, the necessity of having sodium hydroxide in the manometer flasks to absorb the carbon dioxide is a source of error, for the  $H_2S$  formed tends to distil over into it. However, the effects of adding colloidal sulfur are marked, even after 6 hours of shaking, and, moreover, the concentration of colloidal sulfur within limits is without

effect on the reaction, so that it is obvious that a definite effective concentration is present in the solution at all times.

The results can be stated as follows: At pH 7.0 the addition of 0.2 cc. (0.07 mg.) of colloidal sulfur to liver markedly increases the rate of oxygen uptake for about an hour. The acceleration then falls off and an inhibition sets in which lasts for the rest of the experiment. At pH 6.0 the acceleration lasts for from 3 to 4

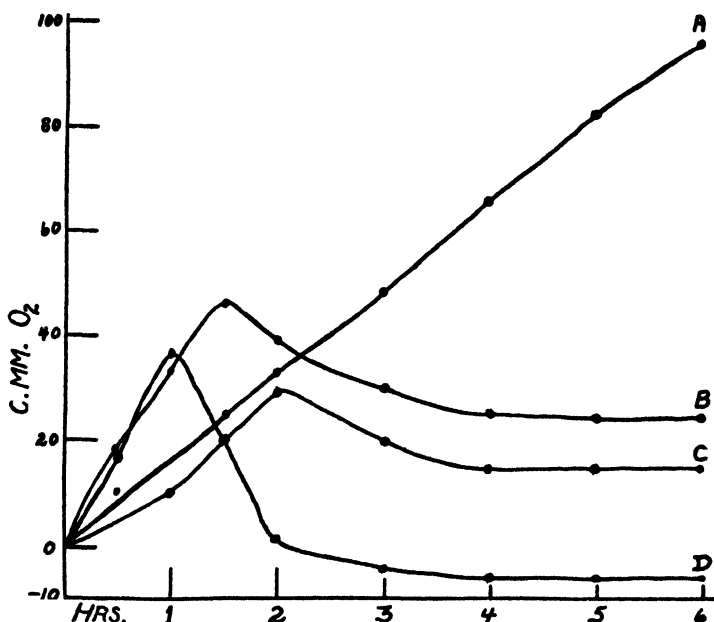


FIG. 1. All curves represent the oxygen uptake of liver plus 0.07 mg. of colloidal sulfur minus the uptake of the respective controls without sulfur. Curve A, pH 6.0 and 7 per cent urethane; Curve B, pH 7.0 and 7 per cent urethane; Curve C, pH 6.0, no urethane; Curve D, pH 7.0, no urethane.

hours followed by an inhibition which is not as marked as at pH 7.0. At both pH's the addition of urethane prolongs the acceleration and at pH 6.0 no inhibition sets in. These results can be duplicated with equivalent concentrations of  $H_2S$ , so that possible different rates of blowing off of  $H_2S$  owing to different rates of formation from colloidal sulfur are not a factor. These results are shown in Fig. 1.

It is obvious that the  $H_2S$  is accelerating one system and inhibiting another. By working at pH 6.0 in the presence of urethane it is possible to study the accelerated reaction, although even then the curve obtained is the resultant of an acceleration and an inhibition. This is shown by the difficulty in getting a liver concentration curve. At times 1 cc. of liver may take up only a little more oxygen than 0.5 cc., showing that the accelerated and inhibited systems are being added in about the same amounts. At other times a fair concentration curve is obtained, but not the theoretical amount based on the assumption that a definite amount of substrate in the liver is being oxidized. For the sulfur itself

TABLE II

*Oxygen Uptake of Liver Plus Urethane and Varying Amounts of Colloidal Sulfur Subtracted from That of Liver Plus Urethane, Showing That Sulfur Concentration Has No Appreciable Effect*

pH 6.0; 30°.

Time	Acceleration of $O_2$ uptake with		
	0.28 mg. colloidal S	0.14 mg. colloidal S	0.07 mg. colloidal S
	<i>c.mm.</i>	<i>c.mm.</i>	<i>c.mm.</i>
30 min.	31	13	6
1 hr., 00 min.	48	34	26
1 " 30 "	63	52	43
2 hrs., 45 "	79	80	69
3 " 45 "	85	92	80
4 " 15 "	89	94	82

is not oxidized, as shown by the fact that amounts of colloidal sulfur from 0.04 to 0.3 mg. do not affect the amount of oxygen taken up. Below 0.04 mg. no effect is seen; above 0.3 mg. inhibition is predominant. This is shown in Table II.

The oxygen uptake of boiled liver is not affected by the addition of colloidal sulfur. Liver washed with water and centrifuged shows little acceleration and only a small inhibition with sulfur. On the assumption that a substrate was present, the oxidation of which was being catalyzed by the presence of sulfur, liver was extracted with water, and the extract boiled and filtered. This, when added to washed liver, increased the oxygen uptake but irregularly, showing that a substrate of an inhibited system was



TABLE III

*Oxygen Uptake of 0.2 Cc. of 2 N Lactic Acid in Presence of 0.5 Cc. of Unwashed Rat Liver at pH 7.0 and 30°*

The total volume of liquid was 2 cc. The experiment was stopped before the oxidation of the lactic acid was complete. The figures represent the oxygen uptake of liver and lactic acid subtracted from the oxygen uptake of the liver alone as control. In each case the control contained all the reagents except the lactic acid.

Time	O <sub>2</sub> uptake; lactic acid	Lactic acid plus		
		0.2 cc. colloidal S	7 per cent urethane	0.2 cc. colloidal S + 7 per cent urethane
	<i>c.mm.</i>	<i>c.mm.</i>	<i>c.mm.</i>	<i>c.mm.</i>
45 min.	15	23	12	6
1 hr., 15 min.	27	33	20	3
1 " 45 "	35	39	26	3
2 hrs., 15 "	47	50	39	2
3 " 00 "	59	60	54	1
3 " 45 "	73	71	73	2

*Oxygen Uptake of 0.1 Cc. of 0.1 N Succinic Acid in Presence of 0.5 Cc. of Washed Guinea Pig Liver at pH 7.0 and 30°*

The conditions were the same as above. Oxidation was much more rapid and inhibition not complete. If more succinic acid is added, inhibition lasts as long as there is succinic acid to be oxidized. After the oxidation of the succinic acid is finished at 132 c.mm., the accelerating effect of the colloidal sulfur can be seen.

Time	O <sub>2</sub> uptake; succinic acid	Succinic acid plus		
		0.2 cc. colloidal S	7 per cent urethane	0.2 cc. colloidal S + 7 per cent urethane
	<i>c.mm.</i>	<i>c.mm.</i>	<i>c.mm.</i>	<i>c.mm.</i>
15 min.	125	103	117	34
25 "	132	139	138	80
35 "	130	144	139	108
1 hr., 00 min.	132	147	138	150
1 " 30 "	130	145	137	155

being added also. An attempt was made to fractionate the boiled extract by adding acetone, filtering off the precipitate, evaporating to dryness, and taking up in buffer. It was found that both sub-

strates were in the acetone-soluble fraction. Further identification has not been made, owing to the extremely minute quantities obtained. Feeding or fasting the guinea pigs 3 to 5 days before killing, in an attempt to increase the acceleration or inhibition, was also without effect. Nor does the addition of substrates such as glucose, alanine, aldehydes, and unsaturated fatty acids have any effect on either the inhibition or acceleration.

*Effect of Colloidal Sulfur and Urethane on Oxygen Uptake of Dehydrogenases*

The inhibition of the oxidation of lactic acid and succinic acid in liver by urethane or colloidal sulfur depends on the amount of enzyme present. Thus with a strong enzyme solution the inhibition of the succinic acid oxidation by 7 per cent urethane is only 7 per cent, and by colloidal sulfur 17.5 per cent. On diluting the enzyme 4 to 5 times, the respective inhibitions are 40 and 27 per cent. The combined action of colloidal sulfur and urethane is, however, much greater than the sum of their separate actions. Thus with an inhibition by urethane of 7 per cent and by sulfur of 17.5 per cent the inhibition of the two together is 73 per cent. Similarly, with the dilute enzyme solution the combined inhibition instead of being 67 per cent is 88 per cent. This phenomenon is much more striking in the case of lactic acid. Rat liver was used for this because it oxidizes lactic acid more readily than guinea pig liver. Urethane alone and colloidal sulfur alone produce a very slight inhibition. Together the inhibition is complete. These effects can be reproduced with equivalent concentrations of  $H_2S$ , so that difference in the rate of formation of  $H_2S$  from colloidal sulfur is not a factor. Table III shows these relationships.

DISCUSSION

It has been shown that colloidal sulfur, *i.e.*  $H_2S$  added to liver, will cause an increased oxygen uptake independent, within limits, of the amount of sulfur added. The nature of the systems thus accelerated is not known. A variety of added substrates was without effect.  $H_2S$  accelerates the action of the proteases (Waldschmidt-Leitz and Purr (13)), but this could not account for the oxygen uptake. But because of this action it was thought

that the acceleration observed here might be due to the action of  $H_2S$  on an oxidative deamination. It has, however, no effect on the oxidative deamination of tyramine by liver (Hare (14)), and, moreover, the tyramine oxidase is inhibited by urethane. The free ammonia formed by liver alone is increased very slightly in the presence of  $H_2S$ , but this deamination is correlated in no way with the oxygen uptake.

Colloidal sulfur and urethane inhibit the dehydrogenases. This accounts in part for the observed inhibition. Urethane alone inhibits the oxygen uptake of liver from 40 to 50 per cent at pH 7.0 and from 70 to 80 per cent at pH 6.0. In the concentrations added it has little effect on the activity of the dehydrogenases present in the concentrated liver preparations used. Therefore its inhibitory effect cannot be entirely due to its action on the dehydrogenases. This is substantiated by the fact that at pH 6.0, where most of the dehydrogenases are partially inactive, the percentage inhibition is greater. It therefore inhibits other oxidizable systems in the liver. It is these last systems which must also be inhibited by  $H_2S$ . This accounts for the fact that the addition of urethane shows up the accelerating effect of  $H_2S$  which otherwise would be counterbalanced by the inhibiting effect.

On the basis of these experiments it is possible to divide the liver enzyme systems into three groups: (1) urethane-stable and accelerated by  $H_2S$ ; (2) the dehydrogenases partially inhibited by urethane and  $H_2S$ , but greatly inhibited by both together; (3) systems inhibited both by urethane and  $H_2S$ .

#### SUMMARY

1. Colloidal sulfur is reduced to  $H_2S$  by liver, and this reduction is accelerated by the presence of urethane.

2. The oxygen uptake of liver is accelerated and later inhibited by colloidal sulfur ( $H_2S$ ). Urethane prevents the inhibition, and the acceleration obtained is independent within limits of the concentration of sulfur.

3. Urethane and colloidal sulfur have separately little effect on the oxygen uptake of the lactic and succinic acid dehydrogenases. Together they cause a marked inhibition.

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## THE PARTIAL DEHYDROGENATION OF OLEANOLIC ACID

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Our previous work on the partial dehydrogenation with sulfur of  $\alpha$ - and  $\beta$ -amyrin,<sup>1</sup> and of certain hederagenin<sup>2</sup> and ursolic acid<sup>3</sup> derivatives has shown that these triterpenes form two groups in regard to their behavior towards this reagent. In the case of  $\alpha$ -amyrin and ursolic acid, apparently 1 mol of hydrogen is removed with the formation of strongly dextrorotating substances which are sulfur-free.  $\beta$ -Amyrin and hederagenin (hederagenic methyl ester), on the other hand, form thio compounds with the simultaneous removal of 3 mols of hydrogen. More recently we have had occasion to extend this reaction to oleanolic acid (caryophyllin) and especially to *oleanolic methyl ester benzoate*. This derivative of oleanolic acid, contrary to the derivative of its isomer, ursolic acid, has been found to yield a dehydrothio compound, *dehydrothiooleanolic methyl ester benzoate*,  $C_{38}H_{48}O_4S$ . This places oleanolic acid in the same category as  $\beta$ -amyrin and hederagenin.

When this thio compound was heated with alcoholic alkali the benzoyl group was readily removed with the formation of *dehydrothiooleanolic methyl ester*. The stability of the methyl ester group possessed by the parent oleanolic methyl ester was retained in this substance, thus paralleling the experience with the thio compound from hederagenic methyl ester. The analogy was further developed by the study of the oxidation of the oleanolic acid derivative with permanganate. The resulting material proved to be a mixture of sulfur-free substances, the separation of

<sup>1</sup> Jacobs, W. A., and Fleck, E. E., *J. Biol. Chem.*, **88**, 137 (1930).

<sup>2</sup> Jacobs, W. A., and Fleck, E. E., *J. Biol. Chem.*, **88**, 153 (1930).

<sup>3</sup> Jacobs, W. A., and Fleck, E. E., *J. Biol. Chem.*, **92**, 487 (1931).

which offered difficulty. This was more readily accomplished after preliminary saponification of the crude mixture with alcoholic alkali. The resulting benzoyl-free material proved to be also methoxyl-free. From the mixture two substances were separated—an *acid* and a *neutral substance*. The acid was characterized by the very sparing solubility of its salts and gave figures corresponding to the formula  $C_{30}H_{42}O_6$  which was confirmed by the analysis of its *methyl ester*. The methyl ester group was still contained in the above crude oxidation mixture but saponification to the acid occurred during the heating with alkali. This was confirmed by the fact that the above ester prepared by remethylation of the acid was readily saponified with 0.1 N alkali. The lability of the methyl ester group of this oxidation product parallels our previous experience with the apparently analogous substance, the so called lactone ester,  $C_{31}H_{44}O_6$ , obtained on oxidation of the dehydrothio derivative of hederagenin methyl ester. In the case of the latter, however, it had been our experience that if strong alkali were used the carboxyl group belonging to the methyl ester group was removed as  $CO_2$  and simultaneously a lactone group was opened to a hydroxy acid. But the above acid,  $C_{30}H_{42}O_6$ , obtained by saponification of the oxidation mixture of dehydrothiooleanolic methyl ester benzoate formed such insoluble salts that we must consider the possibility that the additional steps of  $CO_2$  removal and opening of a possible lactone group were prevented by this physical difficulty.

The second substance which was isolated from the saponification mixture proved to be neutral and also methoxyl-free. In strict analogy with our experience with the oxidation of the thio compounds from hederagenin and  $\beta$ -amyrin this substance should have been a ketone of the formula  $C_{31}H_{44}O_5$ .<sup>4</sup> However, analysis indi-

<sup>4</sup> In the case of the  $\beta$ -amyrin experiments, the ketone formed from its thio compound,  $C_{30}H_{44}OS$ , was found on analysis to have the probable formula  $C_{30}H_{46}O_5$ . In the case of the parallel hederagenin experiments its thio compound,  $C_{31}H_{44}O_2S$ , gave a ketone for which the formula  $C_{31}H_{46}O_4$  was assumed in analogy with the  $\beta$ -amyrin derivative. However, the analyses were in better agreement with a formula  $C_{31}H_{44}O_4$ . A similar situation has now been met in the case of the present oleanolic acid experiments. Because of the analytical results we assume that the hypothetical benzoate formed on oxidation of the thio compound has the formula  $C_{38}H_{48}O_6$  instead of  $C_{38}H_{50}O_6$ .

cated a substance of the formula  $C_{29}H_{42}O_3$ . It is possible, however, that the latter was formed from an intermediate benzoate,  $C_{33}H_{48}O_6$ ,<sup>4</sup> corresponding to the ketones obtained from the hederagenin and  $\beta$ -amyrin experiments. During the subsequent saponification with alkali it is possible that not only the benzoyl group was removed to form a substance,  $C_{31}H_{44}O_5$ , but that the latter further lost carbomethoxyl to form the above substance,  $C_{29}H_{42}O_3$ . The ketone of hedraganic methyl ester origin has already been shown to lose carbomethoxyl when heated with strong alkali. However, contrary to this hederagenin derivative, the assumed carbonyl group in the oleanolic acid compound could not be directly detected by the formation of an oxime.

The formula now accepted for hederagenin is  $C_{31}H_{50}O_4$  and for the amyrins is  $C_{30}H_{50}O$ , but in the case of both ursolic acid and oleanolic acid the formulæ  $C_{30}H_{48}O_3$  and  $C_{31}H_{50}O_3$  have been under discussion.<sup>5</sup> Owing to the difficulty, with such molecules, of deciding between formulæ which differ by only one  $CH_2$  group by analytical means alone, it was hoped that the interconversion of these substances or their derivatives if found possible should help to clarify this question and also to simplify the general problem of the structure of the substances thus grouped together. Since the partial dehydrogenation of hederagenin and oleanolic acid derivatives appeared to take a similar course, an attempt at the correlation of these triterpenes appeared worth the effort on the assumption that each has the same number of carbon atoms. Although the work which follows failed to yield the result desired, the series of substances encountered is recorded for possible future reference.

Both the hederagenin and oleanolic acid molecules contain a carboxyl group and a secondary hydroxyl group. The former possesses an additional primary hydroxyl group. It was hoped that the replacement by hydrogen of the hydroxyls in each substance (preferably as the methyl esters) might yield identical substances. In the case of oleanolic acid its methyl ester was oxidized to the ketone, oleanonic methyl ester. Subsequent reduction by the Clemmensen method gave readily a desoxo derivative. But stereochemical isomerization was simultaneously caused by the

<sup>5</sup> Winterstein, A., and Stein, G., *Z. physiol. Chem.*, **199**, 64 (1931).



acid since the resulting *β*-oleananic methyl ester was levorotatory ( $[\alpha] = -75^\circ$ ) in contradistinction to dextrorotation of the parent hydroxy and keto esters. The formation of *α*-oleananic methyl ester ( $[\alpha] = +71^\circ$ ) without rearrangement was accomplished by heating oleanonic methyl ester semicarbazone with sodium ethylate according to the method of Wolff. A portion of the reaction product was found to be an acid due to the saponifying action of sodium ethylate. On reesterification the resulting methyl ester proved to be a third or *γ*-isomer and different from either the *β*-ester or the expected *α*-ester.

The attempt was then made to prepare the same substance from hederagenin. For this purpose the previously described hederagonic methyl ester<sup>6</sup> was employed. Because it is a *β*-hydroxy ketone it was hoped that it could be converted by dehydration into the unsaturated ketone for further reduction. The resulting material proved to be an unpromising mixture of substances.

Another scheme was then tried in which hederagonic methyl ester was reduced by the method of Clemmensen. The reaction resulted not only in replacement of carbonyl by  $\text{CH}_2$  but the primary hydroxyl group was also removed with the formation of a *desoxodesoxy ester*. The probable explanation of the reaction was simple dehydration which accompanied the reduction. However, all attempts to show the presence of a new double bond in the substance by hydrogenation were unsuccessful. This, however, cannot be regarded as conclusive evidence of the absence of such a double bond. From analysis alone it was difficult to decide between such an unsaturated substance,  $\text{C}_{32}\text{H}_{50}\text{O}_2$ , and one with the formula  $\text{C}_{32}\text{H}_{52}\text{O}_2$ , in which the double bond had been simultaneously reduced. To check this further *hederagonic methyl ester semicarbazone* was heated with sodium ethylate. Two substances resulted—one which proved to be identical with the substance  $\text{C}_{32}\text{H}_{52}\text{O}_2$  obtained by the Clemmensen reduction and the other a substance which would be normally expected, namely the *desoxohydroxy ester*,  $\text{C}_{32}\text{H}_{52}\text{O}_3$ . Apparently under the conditions of the reaction a portion was dehydrated still as the ketone derivative before reduction had occurred, whereas the other portion had been reduced to the desoxo compound before dehydration could

<sup>6</sup> Jacobs, W. A., *J. Biol. Chem.*, **63**, 635 (1925).

occur, and the hydroxy ester was therefore the result. This formed a *benzoate*.

The failure of hydrogenation experiments with the desoxo-desoxy ester leaves uncertain the proper interpretation of the nature of this substance. At any rate it proved to be a substance different from the oleananic methyl esters described above.

#### EXPERIMENTAL

*Preparation of Oleanolic Acid*—A suspension of 500 gm. of ground cloves in 2.5 liters of methyl alcohol and 375 cc. of 2 N KOH was allowed to stand for several days at room temperature. The extract was acidified with acetic acid and then concentrated to a volume of about 750 cc. Crude oleanolic acid which separated was collected and dissolved in a 1 per cent ethyl alcoholic solution of NaOH. After decolorization with Darco the filtrate was diluted with an equal volume of water. On concentration the sodium salt separated. The collected salt was extracted with boiling ether. Decomposition of an alcoholic solution of the salt with dilute hydrochloric acid gave oleanolic acid. On recrystallization from alcohol it formed needles which sintered at 305° and melted at 312° (corrected).

$[\alpha]_D^{25} = +79^\circ$  ( $c = 1.013$  in chloroform)

4.560 mg. substance: 4.290 mg.  $H_2O$ , 13.187 mg.  $CO_2$

4.330 " " : 4.175 " " 12.515 " "

$C_{31}H_{48}O_3$ . Calculated. C 79.08, H 10.71

$C_{30}H_{46}O_3$ . " " 78.88, " 10.61

Found. (a) " 78.88, " 10.53

(b) " 78.83, " 10.79

*Oleanolic Methyl Ester*—The methyl ester was made with diazomethane and melted at 197–198°.

$[\alpha]_D^{25} = +75^\circ$  ( $c = 1.020$  in chloroform)

4.710 mg. substance: 4.535 mg.  $H_2O$ , 13.715 mg.  $CO_2$

4.620 " " : 4.525 " " 13.430 " "

4.350 " " : 2.150 " AgI

$C_{31}H_{48}O_3$ . Calculated. C 79.27, H 10.82, OCH<sub>3</sub> 6.40

$C_{31}H_{46}O_3$ . " " 79.08, " 10.71, " 6.59

Found. (a) " 79.41, " 10.75

(b) " 79.28, " 10.96

(c)

OCH<sub>3</sub>, 6.53

*Oleanolic Methyl Ester Benzoate*—Oleanolic methyl ester was acylated in pyridine solution with benzoyl chloride. It separated from acetone as hexagonal plates which melted at 258–259°.

$[\alpha]_D^{25} = +86^\circ$  ( $c = 1.033$  in chloroform)

4.185 mg. substance: 3.665 mg.  $H_2O$ , 12.165 mg.  $CO_2$

3.973 " " : 3.345 " " 11.565 " "

$C_{33}H_{52}O_4$ . Calculated. C 79.54, H 9.58

$C_{33}H_{52}O_4$ . " " 79.38, " 9.47

Found. (a) " 79.27, " 9.80

(b) " 79.39, " 9.42

#### *Dehydrogenation of Oleanolic Methyl Ester Benzoate*

*Dehydrothiooleanolic Methyl Ester Benzoate*—A mixture of 5 gm. of the benzoate and an equal amount of sulfur was heated in an atmosphere of nitrogen at 235–245° for 2 hours. The dehydrogenation product was extracted with benzene. The concentrated benzene solution was precipitated by addition of methyl alcohol. This crude product was then distilled at 2 mm. The excess sulfur had practically all distilled when the temperature of the metal bath had reached 275°. A light brown resin distilled when the bath temperature was maintained between 300 and 350°. This resin was dissolved in a small volume of benzene. Addition of methyl alcohol caused the deposition of prisms. When the substance was recrystallized from acetone, the melting point was 266–268°.

The cholesterol test gave a reddish brown color which changed to a dull green when the solution was warmed.

$[\alpha]_D^{25} = +32^\circ$  ( $c = 1.053$  in chloroform)

4.182 mg. substance: 3.005 mg.  $H_2O$ , 11.652 mg.  $CO_2$

4.540 " " : 3.345 " " 12.675 " "

5.570 " " : 3.160 "  $BaSO_4$

$C_{33}H_{48}O_4S$ . Calculated. C 76.17, H 8.20, S 5.21

$C_{33}H_{48}O_4S$ . " " 75.95, " 8.06, " 5.33

Found. (a) " 75.99, " 8.04

(b) " 76.14, " 8.24

(c) " " S 5.07

*Dehydrothiooleanolic Methyl Ester*—0.1 gm. of the above benzoate was refluxed in 50 cc. of 5 per cent alcoholic KOH for 4 hours. The product was precipitated with water and then recrystallized

from acetone. It separated as elongated hexagonal plates which melted at 284–285°.

With tetranitromethane a brown color was produced which changed to a yellow when the solution was allowed to stand. Similar colors were given with the thio compounds of hederagenin and  $\beta$ -amyrin.

3.805 mg. substance:	3.060 mg. $H_2O$ ,	10.490 mg. $CO_2$	
4.202 " "	: 3.303 " "	11.583 " "	
3.978 " "	: 1.825 " "	AgI	
8.537 " "	: 4.060 " "	$BaSO_4$	
$C_{32}H_{46}O_2S$ .	Calculated.	C 75.24, H 9.09, OCH <sub>3</sub> 6.07, S 6.27	
$C_{31}H_{44}O_2S$ .	"	" 74.94, " 8.94, " 6.25, " 6.45	
	Found. (a)	" 75.19, " 9.00	
	(b)	" 75.18, " 8.80	
	(c)	OCH <sub>3</sub> 6.06	
	(d)	S 6.53	

#### *Oxidation of the Thio Compound*

*Acid Substance*—To a stirred suspension of 5 gm. of dehydrothiooleanolic methyl ester benzoate in 50 cc. of acetic acid at room temperature were added 150 cc. of a 6 per cent  $KMnO_4$  solution. At the end of 2 hours stirring the solution was diluted with water and the resulting white precipitate was removed from the colloidal solution of  $MnO_2$  by filtration. Attempts to fractionate the mixture of oxidation products were not successful. The crude product was, therefore, saponified by refluxing for 4 hours with 300 cc. of 0.1 N alcoholic KOH. Complete solution took place at the start but as the reaction proceeded a potassium salt separated. The free acid was prepared by addition of dilute HCl to an acetone suspension of the potassium salt and subsequent dilution. Recrystallization of the acid from acetone yielded prisms which melted at 268–269°. It contained no methoxyl.

The cholesterol test gave a yellow color which changed to a deep orange when the solution was warmed. With tetranitromethane no color was produced.

$[\alpha]_D^{25} = -15^\circ$ ( $c = 1.033$ in chloroform)	
4.702 mg. substance:	3.650 mg. $H_2O$ , 12.490 mg. $CO_2$
3.630 " "	: 2.800 " " 9.617 " "
$C_{31}H_{44}O_4$ .	Calculated. C 72.61, H 8.65
$C_{30}H_{42}O_4$ .	" " 72.25, " 8.50
	Found. (a) " 72.45, " 8.69
	(b) " 72.25, " 8.63

*Methyl Ester*—The methyl ester was prepared from the acid with diazomethane. It crystallized from acetone as prisms which melted at 288–289°.

5.173 mg. substance:	4.100 mg. H <sub>2</sub> O,	13.803 mg. CO <sub>2</sub>
4.187 “ “ :	1.980 “ AgI	
C <sub>32</sub> H <sub>48</sub> O <sub>6</sub> .	Calculated.	C 72.95, H 8.82, OCH <sub>3</sub> 5.89
C <sub>31</sub> H <sub>44</sub> O <sub>6</sub> .	“ “	“ 72.61, “ 8.65, “ 6.07
	Found. (a)	“ 72.77, “ 8.87
	(b)	OCH <sub>3</sub> , 6.25

For saponification 14.165 mg. of the ester were dissolved in 2 cc. of alcohol. The solution showed no free acidity. 3.056 cc. of 0.1 N NaOH were added and the mixture was refluxed in an atmosphere of nitrogen for 4 hours, and was then titrated back. Found, 0.263 cc. Calculated for 1 equivalent, 0.276 cc.

*Neutral Substance*—This substance was obtained from the alcoholic mother liquors of the insoluble potassium salt of the previous acid. The precipitate which resulted when this alcoholic solution was diluted with water was dried and crystallized from ether. Recrystallization from acetone yielded needles which melted at 287–288°. Tetranitromethane produced no coloration.

[ $\alpha$ ] <sub>D</sub> <sup>25</sup> = +202° (c = 1.040 in pyridine)	
4.595 mg. substance:	3.920 mg. H <sub>2</sub> O, 13.290 mg. CO <sub>2</sub>
4.000 “ “ :	3.415 “ “ 11.592 “ “
C <sub>30</sub> H <sub>44</sub> O <sub>5</sub> .	Calculated. C 79.59, H 9.80
C <sub>29</sub> H <sub>42</sub> O <sub>5</sub> .	“ “ 79.39, “ 9.66
	Found. (a) “ 78.88, “ 9.55
	(b) “ 79.03, “ 9.55

#### Reduction of Oleanonic Methyl Ester

*Oleanonic Methyl Ester*<sup>7</sup>—Oleanolic methyl ester was oxidized with the required amount of CrO<sub>3</sub> in acetic acid solution. The reaction product was precipitated with water. Recrystallization from acetone yielded needles which melted at 181–182°.

[ $\alpha$ ] <sub>D</sub> <sup>25</sup> = +89° (c = 1.023 in pyridine)	
4.880 mg. substance:	4.495 mg. H <sub>2</sub> O, 14.190 mg. CO <sub>2</sub>
3.855 “ “ :	3.575 “ “ 11.245 “ “
4.435 “ “ :	2.210 “ AgI

<sup>7</sup> Prelog, V., *Collect. Czechsl. Chem. Communicat.*, **2**, 414 (1930).

$C_{22}H_{32}O_2$ .	Calculated.	C 79.60, H 10.45, OCH <sub>3</sub> 6.42
$C_{21}H_{30}O_2$ .	"	" 79.42, " 10.33, " 6.61
	Found. (a)	" 79.30, " 10.30
	(b)	" 79.57, " 10.38
	(c)	OCH <sub>3</sub> 6.57

*β-Oleananic Methyl Ester*—0.2 gm. of oleanonic methyl ester in 50 cc. of acetic acid was refluxed with 5 gm. of amalgamated zinc and 5 cc. of HCl (1.19) for 2 hours. An additional 5 cc. of HCl were added at 15 minute intervals during the course of the reaction. The product obtained by dilution was fractionated from methyl alcohol. A very sparingly soluble fraction in relatively small yield was separated by this procedure and will be described below.

The major fraction was more soluble and consisted mainly of the desoxo ester. The latter formed needles from methyl alcohol which melted at 170–172°.

$$[\alpha]_D^{25} = -75^\circ \text{ (c = 1.000 in pyridine)}$$

3.165 mg. substance: 3.170 mg. H<sub>2</sub>O, 9.510 mg. CO<sub>2</sub>

3.025 " " : 1.500 " AgI

$C_{22}H_{32}O_2$ . Calculated. C 81.98, H 11.19, OCH<sub>3</sub> 6.62

$C_{21}H_{30}O_2$ . " " 81.87, " 11.09, " 6.82

Found. (a) " 81.95, " 11.20

(b)

OCH<sub>3</sub> 6.55

The above sparingly soluble by-product of the reaction formed needles from acetone which melted at 335–337°. The substance contained no methoxyl and gave no color with tetranitromethane.

3.480 mg. substance: 3.320 mg. H<sub>2</sub>O, 10.395 mg. CO<sub>2</sub>

$C_{21}H_{30}O_2$ . Calculated. C 81.87, H 11.09

$C_{20}H_{28}O_2$ . " " 81.74, " 10.99

Found. " 81.46, " 10.68

This substance is probably a desoxolactone. In addition to reduction of the carbonyl group demethylation of the ester group and simultaneous lactonization of the liberated carboxyl group on a double bond has occurred. The lactonization of carboxyl on a double bond in substances of this group has been discussed by Wieland and Hoshino<sup>8</sup> in the case of novaic acid and more recently by Winterstein and coworkers in the case of hederagenin<sup>9</sup>

<sup>8</sup> Wieland, H., and Hoshino, T., *Ann. Chem.*, **479**, 179 (1930).

<sup>9</sup> Winterstein, A., and Wiegand, W., *Z. physiol. Chem.*, **199**, 46 (1931).

and oleanolic acid.<sup>5</sup> In order to check this interpretation of the origin and nature of this lactone the following experiment was made with oleanolic methyl ester.

*Action of Hydrochloric Acid on Oleanolic Methyl Ester*—A solution of 0.5 gm. of carefully purified oleanolic methyl ester in 125 cc. of acetic acid was heated at 110–120° with 20 cc. of concentrated HCl for 5 hours. At 30 minute intervals 20 cc. of additional concentrated HCl were added until a total of 100 cc. of concentrated HCl had been added. The product was precipitated with water. Recrystallization first from methyl alcohol and then from acetone gave a small yield of shining plates which melted at 246–248° with decomposition. The substance was neutral, contained no methoxyl, and gave a yellow color with tetranitromethane. This substance from the analysis is an anhydrolactone and presumably was formed by demethylation of the ester group, lactonization on a double bond, and additional removal of the hydroxyl group to form a new double bond.

2.837 mg. substance:	2.615 mg. H <sub>2</sub> O,	8.497 mg. CO <sub>2</sub>	
	C <sub>31</sub> H <sub>48</sub> O <sub>2</sub> .	Calculated.	C 82.23, H 10.69
	C <sub>30</sub> H <sub>46</sub> O <sub>2</sub> .	"	" 82.12, " 10.58
	Found.	"	" 81.68, " 10.30

When oleanolic acid itself was substituted for the ester in the above reaction a mixture was obtained. On fractionation from ether a small amount of sparingly soluble, higher melting substance was removed, which was probably identical with the acetyl lactone of Winterstein and Stein<sup>6</sup> but which was not further investigated. The main fraction was more soluble and proved to be identical with the above anhydrolactone obtained from the oleanolic methyl ester.

3.838 mg. substance:	3.580 mg. H <sub>2</sub> O,	11.560 mg. CO <sub>2</sub>
	Found.	C 82.14, H 10.44

*Oleanonic Methyl Ester Semicarbazone*—This substance was obtained from the methyl ester by the usual method. Recrystallization from chloroform ether gave needles which melted at 233–235° after preliminary sintering.

3.875 mg. substance:	3.312 mg. H <sub>2</sub> O,	10.368 mg. CO <sub>2</sub>
3.080 " "	: 0.220 cc. N <sub>2</sub> (25°, 763.4 mm.)	

$C_{15}H_{15}O_2N_3$ .	Calculated.	C 73.42, H 9.90, N 7.78
$C_{15}H_{15}O_2N_3$ .	"	" 73.07, " 9.80, " 8.00
	Found. (a)	" 72.97, " 9.56
	(b)	N 8.22

*$\alpha$ -Oleananic Methyl Ester*—A solution of 1.25 gm. of sodium in 30 cc. of absolute alcohol and 0.5 gm. of the above semicarbazone was heated in a tube at  $180^\circ$  for 15 hours. The precipitate obtained on dilution proved to be a mixture of a sodium salt and neutral material. The latter was removed by extraction with ether. The residue from this extract was crystallized from alcohol and formed needles which melted at  $159$ – $160^\circ$ . When mixed with the  $\gamma$ -ester given below a depression was obtained.

$[\alpha]_D^{25} = +71^\circ$ (c = 1.045 in pyridine)
3.767 mg. substance: 3.810 mg. $H_2O$ , 11.350 mg. $CO_2$
4.082 " " : 4.120 " " 12.240 " "
3.373 " " : 1.670 " AgI
$C_{15}H_{15}O_2$ . Calculated. C 81.98, H 11.19, $OCH_3$ 6.62
$C_{15}H_{15}O_2$ . " " 81.87, " 11.09, " 6.82
Found. (a) " 82.17, " 11.32
(b) " 81.78, " 11.29
(c) $OCH_3$ 6.54

*$\gamma$ -Oleananic Acid*—The above sodium salt which remained after extraction of the  $\alpha$ -ester with ether was suspended in ether and brought into solution by addition of a few drops of HCl. The ether solution left a residue which crystallized as hexagonal plates from acetone and melted at  $266$ – $268^\circ$ .

3.963 mg. substance: 3.940 mg. $H_2O$ , 11.920 mg. $CO_2$
4.282 " " : 4.180 " " 12.828 " "
$C_{15}H_{15}O_2$ . Calculated. C 81.87, H 11.09
$C_{15}H_{15}O_2$ . " " 81.75, " 10.98
Found. (a) " 82.03, " 11.12
(b) " 81.71, " 10.92

*$\gamma$ -Oleananic Methyl Ester*—The acid was esterified with diazomethane. The ester from alcohol formed needles which melted at  $168$ – $169^\circ$ .

$[\alpha]_D^{25} = +77^\circ$ (c = 1.035 in pyridine)
3.915 mg. substance: 3.915 mg. $H_2O$ , 11.730 mg. $CO_2$
4.235 " " : 4.160 " " 12.670 " "
3.502 " " : 1.820 " AgI



$C_{32}H_{52}O_2$ .	Calculated.	C 81.98, H 11.19, $OCH_3$ 6.62
$C_{31}H_{50}O_2$ .	"	" 81.87, " 11.09, " 6.82
	Found. (a)	" 81.71, " 11.19
	(b)	" 81.60, " 11.00
	(c)	$OCH_3$ 6.86

*Reduction of Hederagonic Methyl Ester*

*Desoxydesoxomethyl Ester*—A solution of 5 gm. of hederagonic methyl ester<sup>6</sup> in 1000 cc. of acetic acid was refluxed for 1 hour with 125 gm. of amalgamated zinc and 75 cc. of concentrated HCl. An additional 50 cc. of concentrated HCl were added every 15 minutes during the course of the reaction. The material which precipitated on dilution proved to be a mixture of isomers which were separated by recrystallization from acetone. The less soluble material formed prisms which melted at 184–185°.

$[\alpha]_D^{25} = +78^\circ$  ( $c = 1.005$  in pyridine)

3.760 mg. substance 3.660 mg.  $H_2O$ , 11.275 mg.  $CO_2$

3.903 " " : 3.875 " " 11.720 " "

3.603 " " : 1.850 " AgI

$C_{32}H_{50}O_2$ . Calculated. C 82.33, H 10.80,  $OCH_3$  6.64

$C_{32}H_{52}O_2$ . " " 81.98, " 11.19, " 6.62

Found. (a) " 81.78, " 10.89

(b) " 81.90, " 11.11

(c)  $OCH_3$  6.78

If this substance should prove to possess the formula  $C_{32}H_{50}O_2$ , it would then be an anhydridesoxomethyl ester.

The acetone mother liquor from the above  $\alpha$ -hederagonic methyl ester contained more soluble material. After repeated fractionation from alcohol a substance was obtained as needles which melted at 132–135°. It is possible that this substance was still a mixture.

$[\alpha]_D^{25} = -20^\circ$  ( $c = 1.010$  in pyridine)

3.628 mg. substance: 3.645 mg.  $H_2O$ , 10.883 mg.  $CO_2$

4.547 " " : 4.560 " " 13.635 " "

3.235 " " : 1.680 " AgI

Found. (a) C 81.82, H 11.24

(b) " 81.78, " 11.22

(c)  $OCH_3$  6.86

*Hederagonic Methyl Ester Semicarbazone*—This derivative formed needles from chloroform ether which melted at 220–222° after preliminary sintering.

3.942 mg. substance: 3.310 mg.  $H_2O$ , 10.237 mg.  $CO_2$   
 4.047 " " : 0.286 cc.  $N_2$  (25.5°, 757.3 mm.)  
 $C_{12}H_{11}O_4N_2$ . Calculated. C 71.30, H 9.61, N 7.56  
 Found. (a) " 70.83, " 9.40  
 (b) " " N 7.50

*Desoxohydroxymethyl Ester*—1.5 gm. of the semicarbazone were heated in a sealed tube at 180° for 15 hours with a solution of 3.75 gm. of sodium in 90 cc. of absolute alcohol. During the reaction the ester group was saponified. On dilution a mixture of sodium salts was obtained, since a suspension in ether dissolved when acidified. Two acids were found in the ether solution which were fortunately separated by taking advantage of the different solubilities of the ammonium salts as follows. The ether solution was shaken repeatedly with dilute  $NH_4OH$ . The ammoniacal aqueous extract contained one of the substances, the desoxohydroxy acid which was precipitated on acidification. The crude amorphous acid was reesterified with diazomethane. Recrystallization from dilute acetone yielded needles which melted at 165–166°.

$[\alpha]_D^{25} = +84^\circ$  (c = 1.010 in pyridine)  
 4.500 mg. substance: 4.275 mg.  $H_2O$ , 13.085 mg.  $CO_2$   
 4.635 " " : 4.428 " " 13.470 " "  
 3.100 " " : 1.540 " AgI  
 $C_{12}H_{11}O_3$ . Calculated. C 79.27, H 10.82, OCH<sub>3</sub>, 6.40  
 Found. (a) " 79.30, " 10.63  
 (b) " 79.26, " 10.69  
 (c) " OCH<sub>3</sub>, 6.56

This substance gave a benzoate in the usual manner, which crystallized as plates from alcohol and melted at 182–183°.

$[\alpha]_D^{25} = +89^\circ$  (c = 1.045 in pyridine)  
 3.522 mg. substance: 3.015 mg.  $H_2O$ , 10.295 mg.  $CO_2$   
 3.558 " " : 3.003 " " 10.380 " "  
 $C_{11}H_{10}O_4$ . Calculated. C 79.53, H 9.60  
 Found. (a) " 79.71, " 9.58  
 (b) " 79.59, " 9.45

The ether solution which remained after extraction of the desoxohydroxy acid with ammonia contained the salt of a second acid which was recovered by evaporation. The residue formed needles from acetone which melted at 246–247°.

4.143 mg. substance: 4.010 mg.  $H_2O$ , 12.378 mg.  $CO_2$   
 $C_{31}H_{48}O_2$ . Calculated. C 81.87, H 11.09  
 Found. " 81.49, " 10.84

This substance proved to be the free acid of the desoxydesoxo ester obtained by the Clemmensen reduction described above. Methylation with diazomethane gave an ester which melted at  $184-185^\circ$  and corresponded in all properties with the Clemmensen product.

$[\alpha]_D^{25} = +77^\circ$  ( $c = 1.040$  in pyridine)  
 3.893 mg. substance: 3.820 mg.  $H_2O$ , 11.720 mg.  $CO_2$   
 3.474 " " : 3.390 " " 10.458 " "  
 3.575 " " : 1.850 " AgI  
 Found. (a) C 82.11, H 10.98  
 (b) " 82.10, " 10.92  
 (c)  $OCH_3$  6.83

## THE SUGAR OF SARMENTOCYMARIN

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(Received for publication, February 26, 1932)

Sarmentocymarin is a glucoside which has been isolated from *Strophanthus sarmentosus* seeds.<sup>1</sup> On hydrolysis an aglucone, sarmentogenin, was obtained, which was found to belong to the strophanthidin group of cardiac aglucones. At the time the sugar could be obtained only as a syrup. From its reactions it proved to be an  $\alpha$ -desoxy sugar; and from the formulation derived for sarmentocymarin,  $C_{30}H_{46}O_8$ , and for sarmentogenin,  $C_{23}H_{34}O_6$ , it was concluded that the sugar is a methyl ether desoxy sugar with the formula  $C_7H_{14}O_4$  and must therefore be isomeric with cymarose, the methyl ether desoxy sugar of cymarin and periplocymarin. This has recently been substantiated directly.

After several years standing one of the sugar syrups obtained in the earlier work was found to have crystallized. Since then it has been possible to recrystallize the substance which we have called *sarmentose*. After repeated recrystallization from dry ether-petrolie ether it formed either large compact prisms when crystallization was slow or plates on more rapid deposition. It melted sharply at 78–79° and was readily soluble in ether and the usual organic solvents except benzene and petrolie ether. Within 20 minutes after the preparation of the solution the rotation was found to be  $[\alpha]_D^{20} = +12^\circ$  ( $c = 1.08$  in  $H_2O$ ). On standing very slight mutarotation was observed. After 24 hours  $[\alpha]_D^{20} = +15.8^\circ$ .

5.168 mg. substance: 4.070 mg.  $H_2O$ , 9.835 mg.  $CO_2$

4.283 " " : 6.010 " AgI

$C_7H_{14}O_4$ . Calculated. C 51.82, H 8.70, OCH, 19.13

Found. " 51.90, " 8.81

"

OCH, 18.55

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<sup>1</sup> Jacobs, W. A., and Heidelberg, M., *J. Biol. Chem.*, **81**, 765 (1929).



## STROPHANTHIN

### XXV. THE ALLOCATION OF THE LACTONE GROUP OF STROPHANTHIDIN AND RELATED AGLUCONES

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(Received for publication, March 1, 1932)

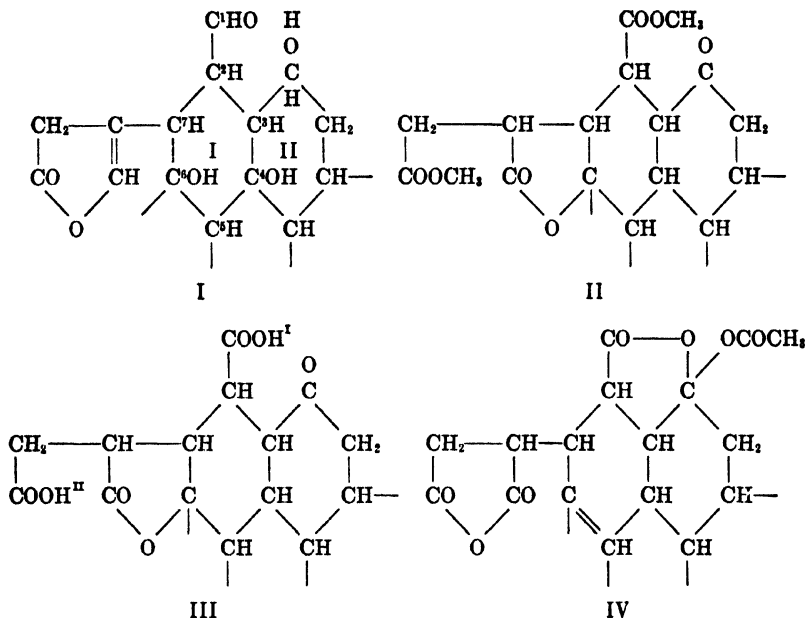
The evidence has already been presented<sup>1</sup> which has shown that the unsaturated lactone group of strophanthidin and therefore of the related cardiac aglucones is a  $\Delta^{\beta,\gamma}$ -lactone side chain attached by its  $\beta$ -carbon atom (Formula I) to a substituted cyclohexane ring, Ring I of these aglucones. In addition the point of attachment to this ring was definitely restricted to carbon atom (5) or (7). At first carbon atom (7) was favored in order to explain the fact that strophanthidin can be isomerized to isostrophanthidin by alcoholic alkali. Since strophanthidinic acid, in which the aldehyde group has been oxidized to carboxyl, could not be isomerized under these conditions—but its ester could be—it was concluded that the isomerization involved a preliminary shift of the  $\Delta^{\beta,\gamma}$  double bond under the influence of a new double bond formed on enolization of the aldehyde or ester carbonyl group. But this argument lost weight as soon as substances such as digitoxigenin, gitoxigenin, and other aglucones<sup>2</sup> which did not have a carbonyl group could also be isomerized by alkali to iso compounds. Therefore, carbon atom (5) also became a possibility. In recent work definite proof has been obtained that carbon atom (7), as originally assumed, is the point of attachment.

After preliminary unsuccessful attempts with certain strophanthidin derivatives our object was accomplished by transformations

<sup>1</sup> Jacobs, W. A., and Gustus, E. L., *J. Biol. Chem.*, **74**, 805, 811 (1927).

<sup>2</sup> Jacobs, W. A., and Gustus, E. L., *J. Biol. Chem.*, **78**, 573 (1928); **79**, 553 (1928). Jacobs, W. A., and Hoffmann, A., *J. Biol. Chem.*, **79**, 519 (1928). Jacobs, W. A., and Heidelberger, M., *J. Biol. Chem.*, **81**, 765 (1929).

made with one of the isomeric ( $[\alpha]_D = +8^\circ$ ) desoxy- $\alpha$ -isostrophanthonic dimethyl esters (II).<sup>3</sup> On saponification this ester yielded the free *dibasic lactone acid* (III). If the point of attachment is carbon atom (7) then the two free carboxyl groups of this dibasic acid as a substituted adipic acid should combine to form an anhydride. The attempt was made to accomplish this by distillation or by heating the acid with acetic anhydride. But no



crystalline substance could be isolated. A more involved procedure became necessary.

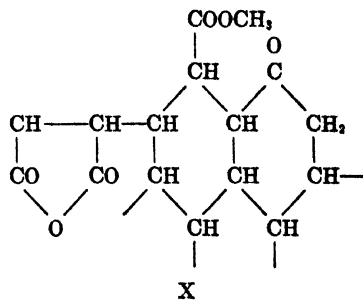
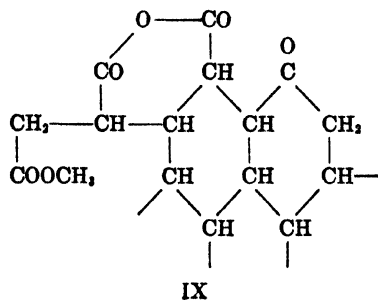
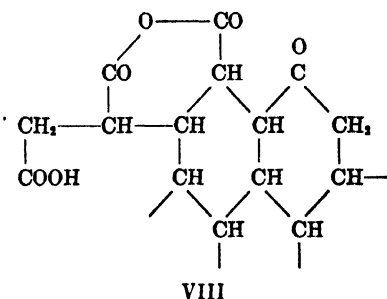
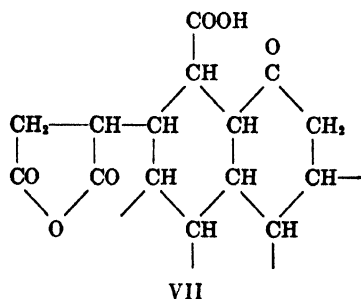
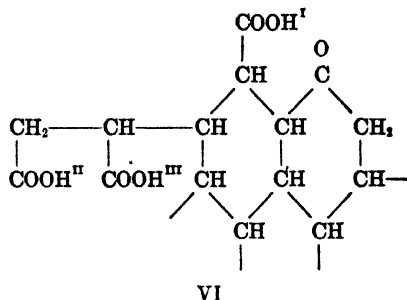
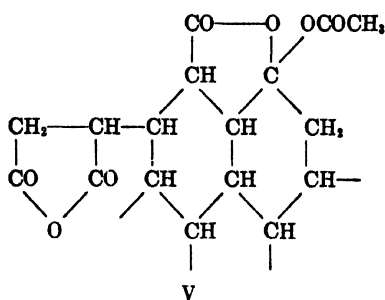
When this acid was heated with a mixture of acetic anhydride and acetyl chloride a crystalline *neutral substance* was obtained which further investigation showed to possess the structure (IV).

The substance owed its origin to the opening up of the lactone group to form a succinic anhydride with simultaneous loss of OH<sup>I</sup> and production of a double bond.<sup>4</sup> At the same time carboxyl<sup>I</sup>

<sup>3</sup> Jacobs, W. A., and Gustus, E. L., *J. Biol. Chem.*, **74**, 820 (1927); **92**, 343 (1931).

<sup>4</sup> Jacobs, W. A., and Gustus, E. L., *J. Biol. Chem.*, **84**, 183 (1929); **92**, 326 (1931).

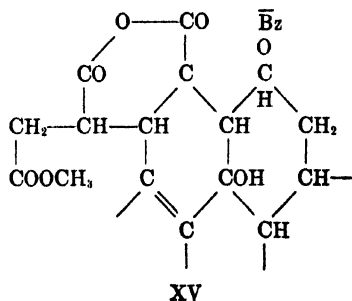
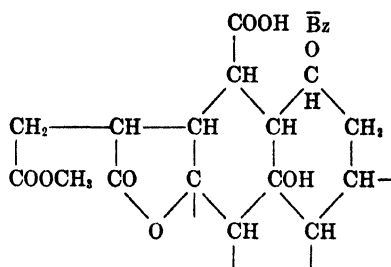
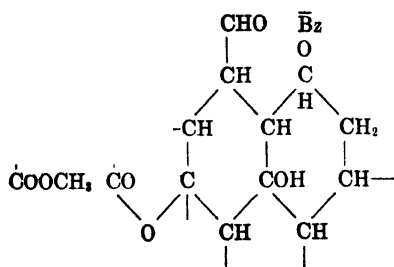
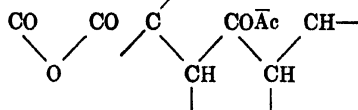
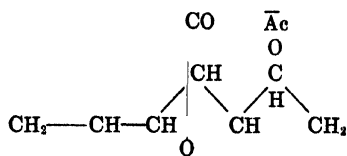
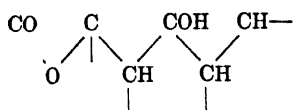
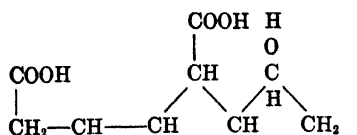
lactonized on the carbonyl group with accompanying acetylation. These facts were determined by a study of its *hydrogenation product* (V) which was formed by the absorption of 1 mol of catalytically activated hydrogen. On saponification with barium hydroxide the



dihydroanhydride was saponified to the *saturated tribasic keto acid* (VI). When the latter was heated *in vacuo* it lost water to form an *anhydride acid* which may be represented by either formula (VII) or (VIII). Although for the purpose in view the decision



between these two possibilities would be immaterial, formula (VII) appears the more probable. When this anhydride acid was very gently treated with 1 per cent methyl alcoholic hydrochloric acid it not only formed a half-ester but simultaneously the carboxyl



which was liberated at once recombined with the remaining free carboxyl to form a new anhydride group (IX). A possible interpretation might be that in the anhydride acid as given in formula (VIII) the free carboxyl was merely esterified. But in no case

has it been our experience with this group of substances that the very gentle treatment used could so readily esterify this carboxyl group. As another interpretation it might be suggested that the free carboxyl group of the anhydride acid given in formula (VII) was esterified with the formation of an anhydride ester (X). But this is again contrary to all experience with the groups involved. In the first place carboxyl<sup>1</sup> in all strophanthidin derivatives in which it occurs has shown considerable resistance to esterification with alcoholic hydrochloric acid. Further, the anhydride group assumed present in formula (X) would certainly open to form an ester in accordance with our general experience with this particular anhydride group in other compounds. In such a case the end result would be the formation at least of a dimethyl ester. Finally, the anhydride ester which was actually obtained was found to be very readily saponified on 30 minutes heating with 0.1 N alkali with the formation of the original tribasic acid (VI). The lability shown by this ester group is a property which is in accord with our previous experience with the esters of this carboxyl group. As a direct check on this conclusion, the *trimethyl ester* of the original tribasic acid was prepared and subjected to identical treatment with 0.1 N alkali. Only a fraction more than 1 mol of alkali was consumed, showing the relative resistance of the remaining ester groups. Formula (IX), therefore, is the only one which satisfies all requirements. The fact that the new anhydride group in this substance as it forms does not in turn react to form a new half-ester is not inconsistent with previous experience. The carboxyls involved are not readily esterified by gentle treatment with alcoholic hydrochloric acid and, in turn, their esters are not readily saponified.

The ready anhydride formation between the carboxyls as given in formula (IX) is explainable only by the positions given to the succinic acid side chain. The unsaturated lactone group of strophanthidin, digitoxigenin, and related aglucones must therefore be attached to carbon atom (7) as given in formula (I).

Before the above series of reactions was performed more obvious attempts were made with  $\alpha$ -isostrophanthic acid itself (XI) which were, however, unsuccessful. When directly heated, this acid lost CO<sub>2</sub> and yielded an amorphous acid substance.<sup>5</sup> Like-

<sup>5</sup> Jacobs, W. A., and Collins, A. M., *J. Biol. Chem.*, **61**, 399 (1924).

wise no crystalline reaction product could be obtained when isostrophanthic acid was heated with acetic anhydride. When acetic anhydride-acetyl chloride was used, however, a small yield of a substance was isolated, which investigation showed to have the probable formula (XII). The same substance was obtained from  $\gamma$ -isostrophanthic acid. Another series of experiments was attempted starting from  $\alpha$ -isostrophanthidic methyl ester benzoate<sup>6</sup> (XIII). On oxidation with permanganate the *benzoate of  $\alpha$ -isostrophanthic monomethyl ester* (XIV) was obtained. When this substance was heated with acetic anhydride-acetyl chloride, instead of the expected unsaturated anhydride (XV) a substance was obtained in very small yield which was apparently identical with a mixed anhydride formed when heated with acetic anhydride alone. Such a substance was, of course, useless for our purpose.

#### EXPERIMENTAL

*Desoxy- $\alpha$ -Isostrophanthonic Acid*—20 gm. of  $\alpha$ -desoxy isostrophanthonic acid dimethyl ester<sup>3</sup> ( $[\alpha]_D = +8^\circ$ ) were warmed with 90 cc. of alcohol and 90 cc. of N NaOH until dissolved. 900 cc. of N NaOH were then added and the mixture was refluxed for  $4\frac{1}{2}$  hours. On acidification to Congo red with HCl the crystalline acid gradually separated. Recrystallized from acetone, in which it was sparingly soluble, the substance formed rhombs which melted at  $260$ – $262^\circ$  with effervescence.

4.563 mg. substance: 2.975 mg.  $H_2O$ , 11.062 mg.  $CO_2$

$C_{25}H_{30}O_7$ . Calculated. C 65.99, H 7.23

Found. " 66.12, " 7.29

*Unsaturated Anhydride Lactone Acetate*,  $C_{25}H_{30}O_7$ —6 gm. of the above acid were heated with 60 cc. of acetic anhydride and 6 cc. of acetyl chloride in a sealed tube at  $80^\circ$  for 16 hours. After concentration under diminished pressure the residue was dissolved in chloroform and the solution was washed free from any acid. The concentrated solution was carefully treated with dry ether. The substance crystallized as irregular platelets or flat prisms which melted at  $245$ – $247^\circ$  with sintering a few degrees lower.

<sup>6</sup> Jacobs, W. A., and Collins, A. M., *J. Biol. Chem.*, **61**, 396 (1924).

4.405 mg. substance: 2.630 mg.  $H_2O$ , 10.953 mg.  $CO_2$   
 $C_{25}H_{32}O_7$ . Calculated. C 67.84, H 6.84  
Found. " 67.82, " 6.68

13.618 mg. of substance were refluxed in 2 cc. of alcohol and 3 cc. of 0.1 N NaOH for 4 hours and then titrated back against phenolphthalein. Calculated for 4 equivalents, 1.232 cc. Found, 1.139 cc.

*Dihydroanhydride Lactone Acetate*,  $C_{25}H_{32}O_7$ —5 gm. of the above unsaturated compound were hydrogenated in acetic acid solution with 0.4 gm. of Adams and Shriner catalyst. Absorption occurred rapidly and the reaction product crystallized during the course of the reduction. After 1 mol of hydrogen had been absorbed, the shaking was interrupted, since further slow absorption continued which involved the anhydride group. The collected material was washed with chloroform in order to dissolve the substance away from the catalyst. The combined filtrates were concentrated to dryness under reduced pressure. From the acetone solution of the residue, when concentrated, the substance was deposited as fine needles. It was very sparingly soluble in the alcohols, somewhat less so in acetic acid and acetone, and readily soluble in chloroform. It melted at 258–259°.

3.617 mg. substance: 2.345 mg.  $H_2O$ , 8.920 mg.  $CO_2$   
 $C_{25}H_{32}O_7$ . Calculated. C 67.53, H 7.26  
Found. " 67.26, " 7.25

*Tribasic Keto Acid*,  $C_{23}H_{32}O_7$ —The saponification of the above dihydroanhydride lactone acetate caused difficulties when conventional attempts were made. The object was achieved by the following procedure.

3 gm. of the substance were heated to boiling in 200 cc. of alcohol. 200 cc. of 5 per cent barium hydroxide solution were added slowly in 20 cc. portions during 1 hour. The refluxing was continued for another hour. During the course of the reaction crystals of the barium salt of the tribasic keto acid were deposited. After chilling, the barium salt was collected with alcohol. It was then vigorously stirred with an excess of dilute sulfuric acid and the precipitate of acid and barium sulfate was collected. The acid was extracted from the barium sulfate with hot acetone, which after concentration was carefully diluted. The acid crystallized

as flat, pointed prisms. After recrystallization from dilute acetone it frothed up at 205–215° and then resolidified to a crystalline substance which finally melted at 270–272°.

4.135 mg. substance: 2.778 mg.  $H_2O$ , 10.000 mg.  $CO_2$   
 $C_{23}H_{32}O_7$ . Calculated. C 65.68, H 7.67  
 Found. " 65.95, " 7.52

11.280 mg. of the acid were titrated with 0.1 N NaOH against phenolphthalein. Calculated for 3 equivalents, 0.806 cc. Found, 0.785 cc.

*Trimethyl Ester of the Tribasic Keto Acid*,  $C_{23}H_{32}O_7$ —The previous acid was esterified with diazomethane. The ester separated from dilute acetone as irregularly shaped plates which melted at 132°.

4.493 mg. substance: 3.310 mg.  $H_2O$ , 11.175 mg.  $CO_2$   
 3.235 " " : 4.800 " AgI  
 $C_{23}H_{32}O_7$ . Calculated. C 67.49, H 8.28,  $OCH_3$  20.12  
 Found. " 67.83, " 8.24  
 "  $OCH_3$  19.61

*Monobasic Keto Acid Anhydride*,  $C_{23}H_{30}O_6$ —1 gm. of the tribasic keto acid,  $C_{23}H_{32}O_7$ , was heated at 210–220° at 1 mm. pressure for 1 hour. Frothing occurred and the molten substance resolidified to a mass of plates. Recrystallized from acetone it formed flat prisms or leaflets which melted at 273–274°.

3.200 mg. substance: 2.190 mg.  $H_2O$ , 8.020 mg.  $CO_2$   
 $C_{23}H_{30}O_6$ . Calculated. C 68.62, H 7.52  
 Found. " 68.35, " 7.65

*Anhydride Keto Methyl Ester*,  $C_{24}H_{32}O_6$ —0.5 gm. of the above keto acid anhydride, which was finely ground, was very gently warmed with 5 cc. of 1 per cent absolute methyl alcoholic HCl solution. Solution occurred within 1 minute and was followed by rapid deposition of the methyl ester. After chilling, the ester was collected with absolute methyl alcohol. After recrystallization from dilute acetone, from which it separated as rosettes of fine needles, it melted at 236–237°. The substance was insoluble in sodium carbonate solution.

4.783 mg. substance: 3.324 mg.  $H_2O$ , 12.175 mg.  $CO_2$   
 4.400 " " : 2.295 " AgI

$C_{26}H_{38}O_6$ .	Calculated.	C 69.19, H 7.75, OCH <sub>3</sub> 7.45
	Found.	" 69.42, " 7.75
	"	OCH <sub>3</sub> 6.89

That this substance did not contain the resistant methyl ester group in which carboxyl (I) is esterified was shown by comparative saponification experiments with this substance and the trimethyl ester,  $C_{26}H_{38}O_7$ .

14.835 mg. of the anhydride ester were covered with 1 cc. of alcohol and 3 cc. of 0.1 N NaOH. The mixture was refluxed for exactly 30 minutes and then titrated back against phenolphthalein. Calculated for 3 equivalents, 1.068 cc. Found, 1.051 cc.

14.895 mg. of the trimethyl ester were subjected to precisely the same treatment. Calculated for 1 equivalent, 0.324 cc. Found, 0.415 cc. or 1.28 mols.

*Action of Acetic Anhydride-Acetyl Chloride on  $\alpha$ -Isostrophanthic Acid*—3.5 gm. of  $\alpha$ -isostrophanthic acid were heated with 30 cc. of acetic anhydride and 3 cc. of acetyl chloride for 16 hours at 80°. After concentration the residue was dissolved in chloroform. After washing, the chloroform solution was concentrated to a small volume and dry ether was carefully added. On standing a very small fraction crystallized. This was collected with ether and recrystallized from acetone. The substance formed almost rectangular platelets and melted at 267–268° with decomposition.

This substance is a  $\gamma$  derivative since  $\gamma$ -isostrophanthic acid gave the same substance.

4.461 mg. substance:	2.615 mg. H <sub>2</sub> O, 10.550 mg. CO <sub>2</sub>
$C_{27}H_{34}O_9$ .	Calculated. C 64.51, H 6.82
	Found. " 64.50, " 6.56

14.123 mg. of substance were refluxed for 2 hours in 2 cc. of alcohol and 3 cc. of 0.1 N NaOH and titrated back against phenolphthalein. Calculated for 4 equivalents, 1.127 cc. Found, 1.100 cc. During this treatment the lactone of carboxyl (I) on OH<sup>I</sup> remained unopened.

In another experiment in which the refluxing was continued for 4.5 hours 14.878 mg. of substance required 1.241 cc. of 0.1 N NaOH. Calculated for 4 equivalents, 1.186 cc.; for 5 equivalents, 1.482 cc.

*$\alpha$ -Isostrophanthic Acid Monomethyl Ester Benzoate*—3.2 gm. of

$\alpha$ -isostrophanthidic acid methyl ester benzoate were turbined with 1.6 gm. of  $\text{KMnO}_4$  in 200 cc. of dry acetone at room temperature. The  $\text{KMnO}_4$  was all used up within 2 hours. The filtrate from  $\text{MnO}_2$  was concentrated to dryness. The residue was taken up in a few cc. of dilute ammonia, the solution was filtered from any insoluble material and then acidified with acetic acid. The partly crystalline material which separated was dissolved in a little acetone. The substance crystallized after dilution as fine rectangular plates which melted at  $261\text{--}262^\circ$  with effervescence.

4.635 mg. substance: 2.905 mg.  $\text{H}_2\text{O}$ , 11.385 mg.  $\text{CO}_2$

5.055 " " : 2.078 "  $\text{AgI}$

$\text{C}_{31}\text{H}_{30}\text{O}_8$ . Calculated. C 67.11, H 6.91,  $\text{OCH}_3$  5.60

Found. " 66.99, " 7.01

"

$\text{OCH}_3$  5.43

12.895 mg. of substance were titrated with 0.1 N  $\text{NaOH}$  against phenolphthalein. Calculated for 1 equivalent, 0.232 cc. Found, 0.240 cc.

*$\alpha$ -Isostrophanthic Acid Monomethyl Ester Benzoate and Acetic Anhydride*—0.1 gm. of the above ester was refluxed for 20 minutes with 10 cc. of acetic anhydride. The residue obtained on evaporation to dryness was recrystallized from chloroform-ether. Needles were obtained which melted at  $220\text{--}221^\circ$  with decomposition.

4.438 mg. substance: 2.755 mg.  $\text{H}_2\text{O}$ , 10.548 mg.  $\text{CO}_2$

$\text{C}_{33}\text{H}_{40}\text{O}_{10}$ . Calculated. C 66.41, H 6.76

Found. " 66.17, " 7.09

When the acetic anhydride-acetyl chloride procedure was used, a very small yield of a neutral crystalline substance was recovered which from melting point and analysis appeared to be identical with the above substance.

## THE RELATION OF GLYCOGEN TO WATER STORAGE IN THE LIVER

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A relationship between the character of the diet and the water content of the body has been recognized for nearly two centuries, but its quantitative aspects are still to be finally determined. A portion of the shift in body water, observed when the composition of the diet is changed, has been attributed to the water accompanying the variation in the quantity of liver glycogen. We have accordingly reinvestigated this question and were preparing to publish our results when the recent paper by Bridge and Bridges (1) appeared.

In 1906, Zuntz, Loewy, Müller, and Caspari (2), basing their calculations on data reported by Pavy (3), concluded that the deposition of 1 gm. of glycogen in the liver was accompanied by the storage of 3 gm. of water. This figure has been in use since, regardless of the fact that the methods employed by Pavy had not reached their present degree of perfection.

### EXPERIMENTAL

In our experiments, albino rats have been subjected to various dietary procedures in order to vary the glycogen content of the liver. In one case, after prolonged fasting, strychnine convulsions were induced to reduce the glycogen to a minimum. The rats were killed, bled, the liver removed immediately, placed in a tared weighing bottle with a ground glass top, and weighed. A portion of the liver was removed and placed in a 60 per cent solution of potassium hydroxide. Glycogen was isolated from this aliquot

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by a modification of Pfüger's method (4), the glycogen hydrolyzed with hydrochloric acid, and the glucose determined by the method of Hagedorn and Jensen (5). The weighing bottle and the remainder of the liver were again weighed to ascertain the weight of the aliquot taken for the glycogen determination, then placed in the freezing unit of a mechanical refrigerator until thoroughly frozen, and dried in a vacuum desiccator to constant weight.

TABLE I  
*Water and Glycogen Content of Livers of Rats in Various States of Nutrition*

Experiment No.	Fresh liver		State of nutrition
	H <sub>2</sub> O	Glycogen	
	<i>per cent</i>	<i>per cent</i>	
1	71.1	0.0	48 hr. fast + 0.9 mg. strychnine
2	70.6	0.02	56 hr. fast
3	70.5	0.18	40 " "
4	69.9	0.23	24 " "
5	70.0	0.38	76 " "
6	70.1	0.93	24 " "
7	70.1	2.14	12 " "
8	70.3	3.54	Undernourished
9	69.7	6.11	Moderately obese; weight 450 gm.
10	70.4	6.15	Maintenance diet
11	63.9	7.62	Extremely obese; weight 700 gm.

### *Results*

Table I contains the percentile values for water and glycogen of the livers of rats on various dietary régimes. In all cases, with the exception of Experiment 11, the water content of the livers was very constant. In this exceptional case, the low water content may well be explained by the presence of striking quantities of fat in the liver. If this one value is omitted, the average water content of the livers was 70.3 per cent, with maximum deviations of +0.8 and -0.6 per cent. The glycogen content of the livers varied from 0 to 7.62 per cent.

## DISCUSSION

In a recent article by Bridge and Bridges (1), the analyses of a number of rabbit livers are reported. It is to be understood from the introduction of the article that they were concerned in proving or disproving the statement that every gm. of glycogen stores 3 gm. of water. In the discussion of their results, however, they deal not with the relationship between the glycogen content and the water stored with it, but with the ratio of the glycogen to the total water of the liver. It is not difficult to understand the reason for the wide divergence of these ratios, since if the ratios were constant, a glycogen-free liver would necessarily be absolutely dry. The presence of a dry liver in a living organism is beyond imagination.

The data on the protein content of the livers can hardly be as valuable as they intimate, since these values were calculated from the total nitrogen content of an organ which is likely to contain considerable and varying amounts of non-protein nitrogen. They also appear to be slightly inconsistent in first showing that there is no relationship between the weight of the liver and the body weight and then computing their data in gm. of each component of the liver per kilo of body weight.

The data obtained by these authors and the data presented in this paper show a remarkable degree of consistency when the differences in technique and animals used are considered. In both cases the percentile water content of the livers was practically constant regardless of the amount of glycogen present. Thus, except in the presence of large quantities of fats, there is a constant ratio between the total water and the total solids of the liver. The average value for this ratio, calculated from our data, is 2.4, or, for each gm. of solids, there are 2.4 gm. of water in the liver. We have varied the percentile value of the glycogen present from 6.15 to 0 without changing the value of this ratio. This seems to indicate a rather constant ratio between the amount of glycogen in the liver and the weight of water held by it. If liver glycogen held more water per gm. than the other solids present, a decrease in the glycogen content would cause a decrease in the ratio of total water to total solids remaining and *vice versa*. But this is not the case. This appears to be ample proof that a gm. of

glycogen stores the same amount of water in the liver as that held by a gm. of the other solids. Thus, when 1 gm. of glycogen is deposited in the liver, 2.4 gm. of water accompany it, and when 1 gm. of liver glycogen is destroyed, 2.4 gm. of water are liberated. This value is remarkably close to that originally proposed by Zuntz, Loewy, Müller, and Caspari (2) when the methods by which their data were obtained are considered.

It is not our contention that all of the shift in body water on varying diets may be accounted for in this manner. Benedict and Milner (6) placed a subject on a very constant physical régime, so that the transformation of energy would be constant from day to day. He was first placed on a diet high in carbohydrate and gained a small amount of weight for 3 days. This gain was nearly entirely due to the retention of water. When this subject was given a high fat diet, isocaloric with the first, he lost over 2700 gm. of water from the body in 3 days. If all of the liver glycogen had been consumed in that period, it could not have accounted for more than 600 to 700 gm. of this water. However, if the source of the water is to be studied, certainly that arising from the destruction of liver glycogen must be recognized, and, as previously stated, both the data from this laboratory and those reported by Bridge and Bridges indicate that this value varies directly with the amount of liver glycogen consumed. We do not care to enter into the question of whether this is "bound water" or "free water."

#### SUMMARY

Glycogen and water have been determined on a number of rat livers. The percentile water content was found to be constant regardless of the glycogen present, except in one case in which the fat content of the liver was high. Thus it is evident that, per unit of weight, the glycogen contains an amount of water equal to that held by the non-carbohydrate solids. This value was found to be 2.4 gm. of water per gm. of glycogen. Any changes in the glycogen content of the liver should be considered, in the study of water balance, as a possible site for the retention of water, or as a source of water to be excreted when liberated by the destruction of glycogen.

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## THE RELATION BETWEEN GLYCOGEN AND WATER STORAGE IN THE LIVER

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It has been known for a long time that the metabolism of large quantities of carbohydrate food is associated with water retention by the body. The usual explanation of this relationship is the statement that glycogen storage in the body is accompanied by the retention of water. Bridge and Bridges (1) have pointed out the inadequacy of the evidence which may be used in favor of this supposition and in an experimental study of the point in question fail to find any relationship between the glycogen content and water storage in the liver. In their Table II they find a rapidly increasing total liver water to total liver glycogen ratio with a falling liver glycogen, a result which is bound to occur whether or not there exists a direct relationship between the liver glycogen and water storage. There is no reason to suppose that all of the liver water is stored with glycogen. It is the *increase in liver water over fasting or an essentially zero glycogen concentration in relation to the increase in liver glycogen* which might be expected to remain constant. The data of Bridge and Bridges are not suitable for this comparison. The method of comparison in which the liver water per kilo of body weight is compared with the amount of glycogen in reference to the same standard (their Table II) is theoretically a suitable one, but only if there is some constancy in the relation between liver weight and body weight in the fasting or glycogen-free state. We know that this relationship almost always shows a high degree of variability. In their Table I Bridge and Bridges have compared the percentage of liver water with the glycogen concentration and find no definite relation. However, what one really wishes to know is whether any water is added to the liver with the glycogen in addition to that present

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when the liver is practically glycogen-free. It must be remembered that if the water concentration of the liver remained constant with increasing glycogen concentrations, it would mean that there was deposited an appreciable amount of water with each gm. of glycogen. We wish to compare the absolute increase in liver

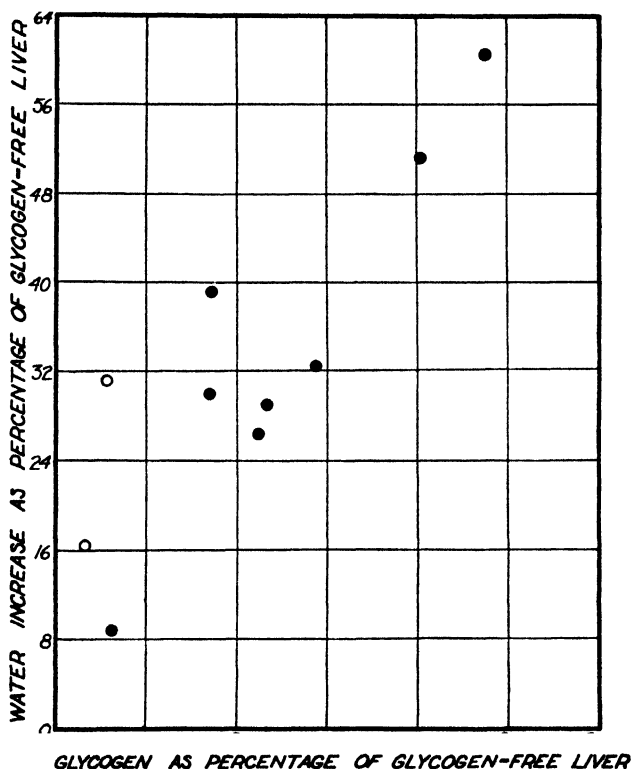


FIG. 1. Relationship between glycogen and water storage in the liver. (Data of Bridge and Bridges (1).)

glycogen with the absolute increase in liver water above that contained in a glycogen-free liver. This may be done by comparing the glycogen and added water as percentages of the glycogen-free liver. We have treated the data of Bridge and Bridges in this fashion in our Fig. 1. The average of their last two figures, 69.5 per cent, was used as the water concentration of a glycogen-

free liver. Their other low glycogen liver water figure was excluded because of the extraordinary diet the rabbit had received. Plotted in Fig. 1 there is a very suggestive relationship between the increase in the water and glycogen concentrations of the liver. That it is not more definite we believe due to the diverse conditions under which these investigators made their observations. It seems quite reasonable to assume that there may be many factors other than the glycogen content of the liver which determine the amount of water present, and with the animals receiving different methods of treatment there was an excellent opportunity for them to come into operation in this instance.

It has seemed desirable to reinvestigate the relationship between water and glycogen storage in the liver under conditions in which an attempt could be made to avoid changes in the water reserves other than those produced by glycogen storage. Since it seemed doubtful that the liver alone could account for all of the fluid producing the loss in weight when the diet is changed from one predominantly carbohydrate to one of fat (2), determinations of the water content of skeletal muscle and incidentally of glycogen in this tissue were also made.

#### EXPERIMENTAL

Twenty-two young rabbits were fasted for 24 hours. Rabbits 1 and 2 were each given 0.5 mg. of adrenalin and killed in an hour. Rabbits 3 and 4 were killed at the end of the fasting period. Rabbits 5 to 12 were all given 15 gm. of glucose per kilo of body weight by stomach tube. Rabbits 5 and 6 were killed in 15 minutes, Rabbits 7 and 8 in 30 minutes, Rabbits 9 and 10 in 45 minutes, and Rabbits 11 and 12 in 60 minutes; Rabbits 13 to 22 were given 15 gm. of glucose per kilo and 10 gm. per kilo per hour thereafter. Rabbits 13 and 14 were killed in 90 minutes, Rabbits 15 and 16 in 120 minutes, Rabbits 17 and 18 in 150 minutes, Rabbits 19 and 20 in 180 minutes, and Rabbits 21 and 22 in 240 minutes. Rabbits 23 to 26 were treated in the same manner but they had not been previously fasted. Rabbit 23 was killed in 5 hours, Rabbit 24 in 7 hours, Rabbit 25 in 9 hours, and Rabbit 26 in 12 hours after the first dose of glucose. Rabbits 27 and 28 were not fasted and had been on a diet of dried carrots for 15 days, a régime which we have found to be very efficacious in producing



high concentrations of liver glycogen. Rabbits 29 and 30 also had been receiving the dried carrot diet and were then given 15 gm. of glucose per kilo and killed 6 hours after. All glucose was given by stomach tube in 20 and 30 per cent concentration.

The animals were killed and the samples removed in the manner described by Sahyun and Luck (3). The glycogen determinations were made by the method described by Sahyun (4), the Shaffer-Hartmann (5) procedure being used for determining the amount of glucose. Our glycogen figures are expressed in terms of glucose. The water content of the liver and muscle was determined by drying samples to constant weight *in vacuo* at 80°.

#### DISCUSSION

Our results are presented in Table I. The relative constancy in the water content with increasing glycogen concentrations demonstrates that water is stored with the glycogen. This is shown in Fig. 2 by plotting the glycogen and water increase as percentages of the glycogen-free liver. For the percentage of water in the glycogen-free liver we used the average, 71 per cent, of our lower glycogen figures.

Our group of rabbits must have had a relatively constant liver weight to body weight relationship during fasting for there is a definite relation in Table I (Columns 3, 4, and 6) between liver weight in relation to body weight and the glycogen concentration, and in Fig. 3 between the absolute amounts of liver glycogen and water in relation to body weight. In spite of our attempt to maintain uniform conditions the relationship is not a great deal better than that found (Fig. 1) with the data of Bridge and Bridges. There are two possible reasons for this. One concerns the technical difficulty of obtaining specimens of liver tissue either relatively free from or with a constant percentage of blood. We made some attempt to control this by rapidly blotting on filter paper the excised bits of tissue used for analysis, but similar blotted slices of liver which we left a few minutes were soon surrounded with an appreciable amount of oozed blood. Another factor which undoubtedly contributes to the production of irregular results is the fact that the maintenance of truly constant conditions is practically impossible. The various procedures necessary for producing changes in the glycogen concentration of the liver are

in themselves alterations in the experimental conditions. The glycogen content of the skeletal muscle underwent no regular variation nor is there any relation between the water concentration

TABLE I  
*Water and Glycogen Concentrations in Liver*

Rabbit No. (1)	Body weight (2)	Liver weight (3)	Liver per kilo body weight (4)	H <sub>2</sub> O in liver (5)	Glycogen in liver (6)	Gm. water per gm. glycogen (7)	H <sub>2</sub> O in muscle (8)	Glycogen in muscle (9)
	kg.	gm.	gm.	per cent	per cent		per cent	per cent
1	1.36	31	23	72.1	0.19		77.0	0.12
18	1.00	41	41	73.5	0.23		75.0	0.04
4	1.09	44	40	71.7	0.25		78.0	0.14
2	1.24	38	31	73.7	0.29		78.3	0.03
14	1.20	46	38	67.6	0.37		74.0	0.01
6	1.35	41	30	71.3	0.39		78.0	0.21
24	1.78	68	38	72.1	0.44		70.8	0.10
17	1.45	52	36	71.2	0.64	3.6	73.2	0.20
7	1.56	42	27	72.4	0.68	9.0	77.3	0.27
20	1.50	55	37	66.0	0.69	0.0	72.0	0.06
9	1.52	64	42	69.2	0.86	0.6	76.0	0.37
19	1.60	59	37	72.3	0.89	7.5	73.3	0.08
3	1.46	42	29	70.5	0.89	0.5	79.4	0.08
10	1.38	55	36	72.1	0.98	8.1	76.4	0.13
5	1.65	64	30	72.4	0.98	7.3	77.8	0.26
8	1.32	59	33	71.0	1.41	2.4	77.4	0.15
12	1.28	42	33	71.7	2.13	3.5	76.7	0.23
27	1.49	39	27	70.6	2.26	1.9	79.0	0.13
11	1.45	43	30	70.6	2.40	1.9	77.0	0.08
13	1.25	54	43	72.4	2.50	4.4	75.6	0.01
15	1.60	62	39	67.3	5.50	0.2	75.3	0.18
16	1.20	47	39	67.5	5.80	0.4	76.4	0.13
30	1.81	52	29	72.1	6.10	3.1	77.4	0.25
29	1.80	66	37	70.4	9.40	2.2	75.0	0.55
22	1.20	58	48	68.5	9.40	1.5	71.4	0.21
21	1.40	58	41	67.5	9.60	1.3	71.6	0.04
28	1.85	78	42	70.8	9.60	2.4	75.4	0.52
23	1.58	71	45	67.8	12.20	1.7	72.1	0.41
26	1.50	75	50	69.7	13.10	2.1	73.5	0.62
25	1.50	83	55	70.7	13.30	2.4	72.7	0.76

in this tissue and the glycogen storage in the liver unless it be a tendency to an inverse one, which gives no hint as to the site of water storage on high carbohydrate diets.

We have demonstrated here a definite relationship between glycogen and water storage in the liver, a finding in agreement with our interpretation of the data of Bridge and Bridges (1). Do these results substantiate the old idea (6) that water is stored with the glycogen in the ratio of 3:1? Our average figures (Table I) give 2.96 gm. (since we have expressed our glycogen in terms of

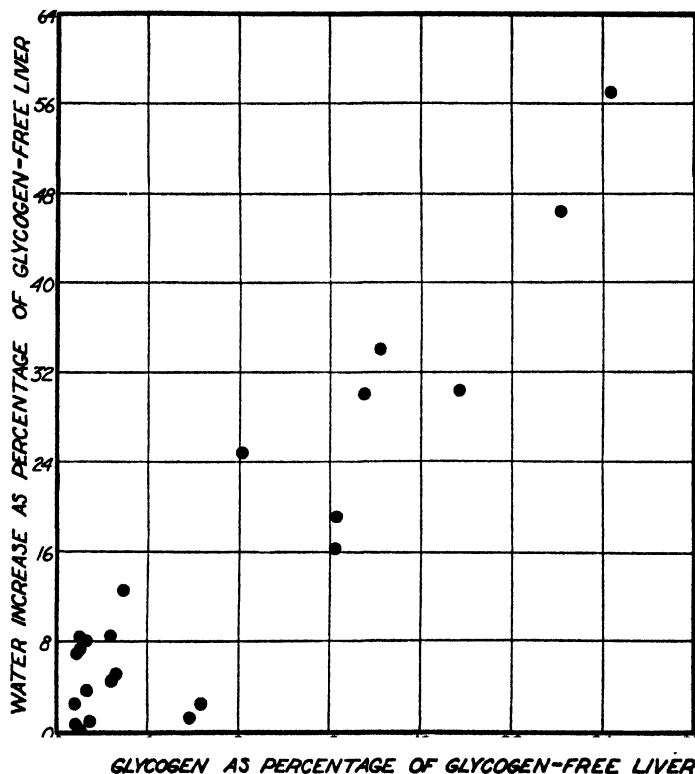


Fig. 2. Relationship between glycogen and water storage in the liver

glucose, these water figures should theoretically be multiplied by 0.927) of water per gm. of glycogen, but it can be seen from Fig. 2 that this is due to the high ratios found with the less reliable low glycogen livers and that 2 gm. of water per gm. of glycogen is more nearly correct. The data of Bridge and Bridges (Fig. 1) give an average of 5.63 gm. of water per gm. of glycogen, but, if

we omit the last two very diverse figures, and fit the observations in Fig. 1 by inspection, we find 3.1 gm. of water to 1.0 gm. of glycogen more appropriate. In both sets of data the actual glycogen to water ratio is dependent on the water concentration which is used for the glycogen-free liver. Our data on this point are rather meager. Should we have used the 69.5 per cent of Bridge and Bridges in place of the 71.0 per cent which was used

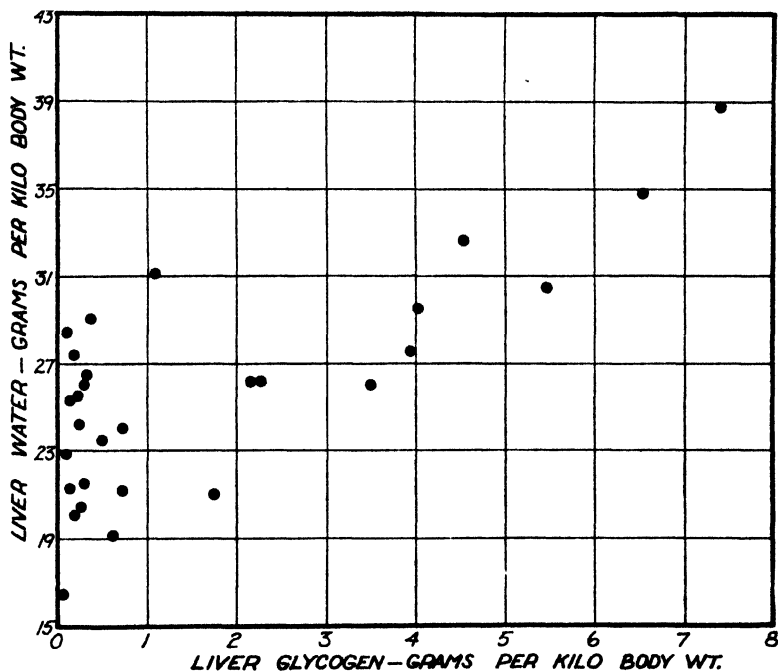


FIG. 3. Glycogen and water storage in the liver in relation to body weight.

for our data, the result would have been about 3 in place of 2 gm. of water per gm. of glycogen, which coincides very well with the 3.1 gm. of their data. Although in the absence of more adequate knowledge of the water content of the glycogen-free liver we are unable to determine definitely the amount of water stored with glycogen, we may conclude that our results do not oppose the frequently quoted statement that with every gm. of glycogen 3 gm. of water are stored.

## SUMMARY

There is a tendency for water to be stored in direct proportion to glycogen storage in the liver. Evidence of many other factors than glycogen storage which affect the water reserves of the liver is found in the variability of the *increased glycogen to increased water* relationship in the rabbit liver under apparently constant conditions. The results obtained, although they cannot directly support it, do not oppose the frequently quoted statement that with every gm. of glycogen 3 gm. of water are stored.

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# THE RELATION OF GLYCOGEN TO WATER STORAGE IN THE LIVER

A REPLY TO THE COMMUNICATIONS OF PUCKETT AND WILEY AND OF  
MACKEY AND BERGMAN

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In a recent article in this *Journal* (1) we reached the conclusion that it was not permissible to express the amount of water stored with glycogen by an exact mathematical ratio. Puckett and Wiley (2) and MacKay and Bergman (3) are now presenting data somewhat similar to our own, from which they conclude that each gm. of stored glycogen is accompanied by an amount of water which is essentially the same as that proposed by Zuntz *et al.* in 1906 (4). This discrepancy of views deserves clarification.

As Puckett and Wiley point out, one could not expect the ratio of glycogen to total liver water to be arithmetically constant, for a glycogen-free liver would then be water-free also, an obvious absurdity. However, because present methods do not permit a direct study of the water bound with glycogen, one is forced to utilize either percentage of water, or total liver water in formulating any deductions. Both criteria were considered in our previous report, emphasis being placed on the latter because of its employment by Zuntz and his associates in the original work on the subject.

Puckett and Wiley present experimental data on a series of eleven rats in which they found the percentage of liver water to exhibit a high degree of constancy, while the glycogen varied from 0 to 7.62 per cent. They argue that since the sum of all liver solids holds 2.4 gm. of water per gm. of solids  $\left( \frac{70.3 \text{ (per cent H}_2\text{O)}}{29.7 \text{ (per cent solids)}} = 2.4 \right)$  therefore, glycogen, being one of them, must hold this same pro-

portion of water. Such an argument would be valid only (1) if protein, fat, salts, and in fact each one of the other liver solids caused exactly this proportion of water to be stored, or (2) if, in case the other liver solids bind varying amounts of water, the sum of their hydration properties alters with each change in the concentration of glycogen so as to maintain exactly the ratio of 2.4 gm. of water for each gm. of non-glycogen solids. The first of these possibilities has never been established; moreover, it has been repeatedly shown that, whereas the storage of protein and of minerals is accompanied by a large amount of water, the retention of fat binds little more water than could be accounted for by the supporting connective tissue. The second possibility is an obvious absurdity. It is, thus, apparent that the argument used by Puckett and Wiley is not justified under the condition of their experiment.

Based on the results of a series of measurements on rat livers, Puckett and Wiley postulate a constant percentage of liver water irrespective of wide variations in glycogen. In five of their eleven rats it will be noted that the liver glycogen content was below 0.4 per cent. If the contention of these authors were true and each gm. of glycogen held 2.4 gm. of water, such minute variations in glycogen would, at the most, have produced a change in water content of less than 1 per cent, which is well within the experimental error. For practical purposes, therefore, the glycogen of these livers must be considered constant. The thesis, thus, rests upon the remaining six experiments, one of which, with a liver water content of 63.9 per cent, they have arbitrarily excluded from the series. One may well raise the question as to how constant the percentage of liver water would have been had the authors studied a larger series of animals. The answer to this question is to be found in the data of our experiments, of those of MacKay and Bergman (3), of Lowrey (5), of Profitlich (6), and of others who have analyzed the water content of animal livers. All of these workers have found considerable variation in individual animals. This fact is illustrated graphically in Chart 1 where we have plotted our own data on rats and rabbits, the figures of Puckett and Wiley on rats, and the results of MacKay and Bergman with rabbits. While in the majority of animals the water content is not far from 70 per cent, variations are met with between

62 and 75 per cent. Unless such variations from the average are completely ignored, values could be calculated, even if the method of estimation of Puckett and Wiley were permissible, varying 30 per cent in both directions. It is, therefore, apparent that neither the premise nor the argument of these authors rests upon a firm foundation.

MacKay and Bergman (3) have made a study of the question under discussion, proceeding in a manner entirely similar to our

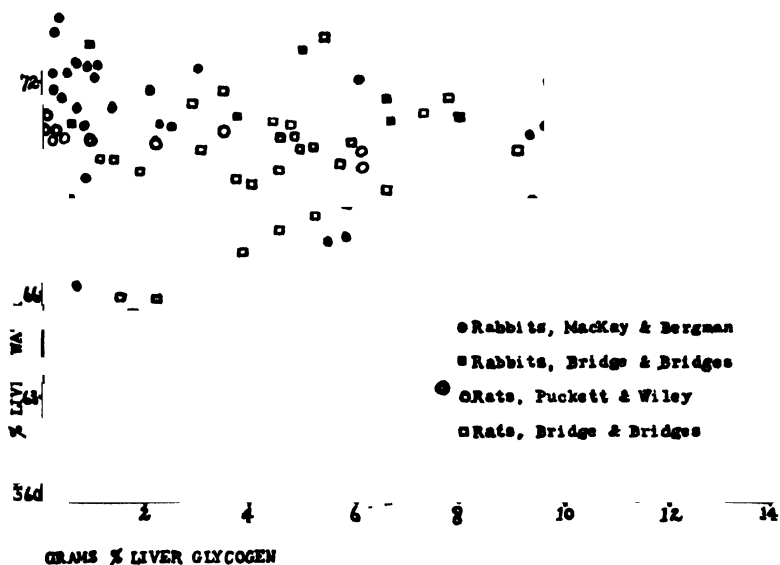


CHART 1. Showing the percentage of liver water (ordinate) plotted against the percentage of liver glycogen (abscissa).

own. Their method of calculation, based on the increase of liver water over and above that contained in a glycogen-free liver, is theoretically, we believe, the only correct approach to the problem. Examination of the data presented by these authors will show that our findings in regard to the limits and variability of the concentration of liver water and glycogen are entirely confirmed. Only in the interpretation to be placed on the findings does the difference in opinion occur.



A cursory perusal of the results of MacKay and Bergman gives one the impression that a relationship between glycogen and water in the liver has been satisfactorily established. However, on more careful examination of the experimental results and discussion, the relationship seems less clearly defined. In the first place, if such a correlation exists, it should be demonstrable in their Table I (Columns 4 and 6) and Fig. 3 in which the experimental facts are featured exclusively, uncomplicated by the somewhat questionable method of calculation used in Figs. 1 and 2. Examination of Fig. 3 will show that in their low glycogen livers variations were present between 16.5 and 30.9 gm. of water per kilo of body weight (the latter figure has been omitted from Fig. 3). Of the higher glycogen livers only five points on Fig. 3 extend outside of this range. It should also be noted that these five points were obtained in animals subjected to distinctly abnormal procedures. The amount of water administered was comparable in quantity to that used by Rowntree (7) to produce a moderate degree of water intoxication in rabbits. When there is added to excessive water intake prolonged administration of large amounts of glucose, the results of the experiments become increasingly difficult to evaluate. Conceivably, the excess of water in these high glycogen livers might be an expression of water intoxication, and not necessarily an indication of the water-binding properties of glycogen. But regardless of this uncertainty, if the attempt is made to formulate a linear relationship between the points of Fig. 3, one is confronted with the dilemma of deciding on a point of origin, since the variations at the origin cover a large extent of the ordinate. The slope of the line, and hence the ratio in question, would depend wholly on the origin chosen.

MacKay and Bergman have used the average figure of 71.0 per cent water in their low glycogen livers as the basis for all their calculations and in the formulation of their conclusions. Upon the accuracy of this figure stands or falls the value of their entire argument. That the authors themselves feel uncertain about this basis is illustrated by their discussion. If their average figure of 71.0 per cent water (actually 71.7) as the correct composition of a glycogen-free liver is used, their calculations show the series of rabbit livers to average 2.96 gm. of water per gm. of glycogen. However, because of recognized errors they believe

2 gm. of water per gm. of glycogen to be more nearly correct. If they use the water content of two out of three of our low glycogen livers as a basis, they again obtain the figure 3 gm. for their more trustworthy experiments. Our own experimental data are brought into agreement with this finding by excepting two more of our animals. In reality the true average of our experiments was 5.63, with a range of 2.9 to 14.3 gm. Incidentally, it should be noted that their own animals varied from 0.0 to 9.0 gm. of water per gm. of glycogen, these limits occurring in rabbits having identical concentrations of liver glycogen. Obviously, in spite of the best possible approach to the problem, the conclusion depends on whether one uses as a basis for calculation the average results of all available experiments or a few carefully selected ones. While these authors conclude that their studies do not oppose the statement that every gm. of glycogen stores with it 3 gm. of water, we feel that their evidence supporting the statement is entirely meaningless when considered in the light of the selective methods used and the wide variations found.

Obviously, as MacKay and Bergman suggest, factors other than glycogen must be operating in regulating the water content of animal livers, for otherwise a low glycogen liver would contain a constant minimum of water. In all probability it is the presence of these other unknown factors which so shrouds the water-binding properties of glycogen alone. It seems only reasonable to suppose that the liver water is controlled by the same physicochemical mechanism which regulates the rest of the body water, probably with a glycogen factor in addition. Until factors other than glycogen are better understood and more completely controlled, we believe it to be premature to attempt an exact mathematical formulation of the relation of liver water to liver glycogen alone. Herein lies the essential difference between our own conclusion and that of the authors mentioned above.

It has not been our aim to deny entirely the claim of Zuntz *et al.*, of Puckett and Wiley, and of MacKay and Bergman, that glycogen is a factor in holding water in the liver. It is quite inconceivable that glycogen could be stored in the body in a dry state. It is generally accepted that proteins and salts are held in certain definite concentrations in the body; and in all probability glycogen, fats, phospholipids, etc., are also fixed with water.

However, we should like to reaffirm our original contention: that, from the data available, it is unjustifiable to define an exact mathematical relationship between the storage of glycogen and water in the liver, and that the water bound to glycogen is not of sufficient magnitude to explain the tremendous shifts in body water that occur with changes in the proportions of fat and carbohydrate in the diet. To accept a mathematical expression as the explanation of the phenomenon, as has been done in the past, tends to close the door upon further investigation of the matter. If the recent discussion has succeeded simply in pointing out the fallacies and dangers of the present orthodox conception, and has indicated the way toward a more sound physiological approach, then it has achieved its primary purpose. What the exact mechanism of the phenomenon is, will only be clarified by careful investigations of the future.

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## THE EFFECTS OF CAROTENE AND OF VITAMIN A ON THE OXIDATION OF LINOLEIC ACID

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Following the observation that carotene is present in medullated nerve (Monaghan and Schmitt (1)) experiments were made to determine the possible significance of this substance in the metabolic processes of nerve. In the course of this work, certain *in vitro* experiments were carried out to test the effect of carotene on the oxygen uptake of unsaturated fatty acids. The results of these experiments seem sufficiently interesting to warrant description at this time without necessary regard to any possible significance in nerve.

Mattill (2), Olcovich and Mattill (3), and Olcott and Mattill (4) studied the effect of a number of substances on the induction period of unsaturated fats. They concluded from their data that most of the substances tested, including the unsaponifiable material from cod liver oil, have little if any effect on the induction period of a mixture of lard and cod liver oil. Certain compounds, however, including carotene, act as prooxidants, shortening the induction period, while a few substances act as antioxidants, lengthening the induction period. Their observations refer only to the induction period and do not pertain to the subsequent period of rapid oxidation. Our own experiments, which concern themselves only with the period of rapid oxygen consumption following the induction period, yield results on carotene and cod liver oil extract which are not in accord with the interpretations of Mattill and coworkers. In our opinion an analysis of the action of a substance may better be obtained from a procedure which allows of continuous readings from the beginning of the experiment than from a method such as that described by Mattill (2). In measuring the pro- or antioxidant activities of a substance

which itself is subject to oxidation, it is essential to distinguish between the effects of the original substance and those of the oxidized form. Measurements on the length of the induction period, especially when this period lasts for many hours, may yield little information on the action of the original substance which may have been partially or completely oxidized in the course of the experiment.

#### EXPERIMENTAL<sup>1</sup>

The carotene used in this work was obtained from the British Drug Houses and had a melting point of 172–173°, as determined in closed tubes. Immediately upon removal from the original sealed, evacuated vials, the crystals are bright red. This color fades to a light yellow upon exposure to air. The decolorization requires several weeks at room temperature but may be brought about within a few hours at a temperature of 105°. According to Olcovich and Mattill (3) fading takes place as readily in the absence of oxygen as in its presence and is to be ascribed to an intramolecular rearrangement of the carotene molecule rather than to an oxidation. Nevertheless it is certainly true that carotene may become oxidized and that this oxidation is accompanied by a decolorization (Arnaud (5), Willstätter and Miegl (6), Willstätter and Escher (7), and von Euler (8)). Carotene dissolved in linoleic acid becomes decolorized within a few hours when exposed to air but no detectable decolorization over a period of months occurs if the mixture be placed in sealed tubes in an atmosphere of nitrogen. Decolorization may be brought about apparently by a variety of agents but it is probable that the resulting products are not identical.

The unsaturated fatty acid used in most of the present experiments was linoleic acid (Kahlbaum's purest; iodine number, 180). This acid was used because its induction period is short and the oxygen uptake following the induction period is very rapid. The routine procedure was to expose the linoleic acid to the air for some time before the experiment to insure a rapid consumption of oxygen. Simultaneous measurements were made in Warburg

<sup>1</sup> These experiments were performed during the summer of 1931 and were subsidized in part by a grant to Washington University by the Rockefeller Foundation.

respirometers of the oxygen uptake of linoleic acid alone, of linoleic acid plus carotene, and of linoleic acid plus oxidized carotene. In fourteen experiments it was shown that carotene strongly inhibits the oxidation of the acid; the average degree of inhibition for the 1st hour was 51 per cent. The typical curve shows a strong inhibition in the 1st few hours which wears off as the carotene becomes bleached, at which time the carotene, now presumably oxidized, actually increases the oxygen uptake of the acid. The rate of escape of inhibition depends among other factors upon the concentration of the carotene used and on the original rate of oxidation of the acid.

To obtain oxidized carotene suitable for use in these experiments carotene crystals were dissolved in caproic acid and exposed to air until the color of the solution changed to a light yellow. Proper control of this use of caproic acid as a vehicle for the carotene was exercised by adding the same amount of caproic acid to the linoleic acid contained in the control vessel of the differential manometers used in these experiments. Any effects which might have arisen from the caproic acid were thus balanced out.

In twelve experiments the oxidized carotene accelerated the oxygen uptake of the linoleic acid from the very start, the average acceleration in the 1st hour being 28 per cent. Fig. 1 represents a typical experiment showing the effect of carotene and of oxidized carotene on the oxygen uptake of linoleic acid. The experiment was performed in differential manometers. Curves C and B represent the oxygen uptake of carotene and of oxidized carotene respectively in the absence of linoleic acid. Curves D and A demonstrate the effect of carotene and of oxidized carotene respectively on the oxygen uptake of linoleic acid. In the latter case the control vessels of the differential manometers received the same amount of linoleic acid as the experimental vessels, the only difference between the contents of the control and experimental vessels being the presence of carotene and of oxidized carotene in the experimental vessels for which the data are given in Curves D and A respectively. It is apparent that while carotene inhibited the  $O_2$  uptake of the acid (Curve D), the oxidized carotene accelerated its oxidation (Curve A). Similar results were obtained in one experiment in which linseed oil was substituted for linoleic acid.

As stated above, carotene may be bleached by heating at 105° for 3 hours. In two experiments it was found that carotene so bleached not only failed to accelerate the oxygen uptake of linoleic acid, but actually inhibited the uptake somewhat, indicating clearly that this substance behaves very differently from the carotene which has been allowed to bleach at room temperature in caproic acid.

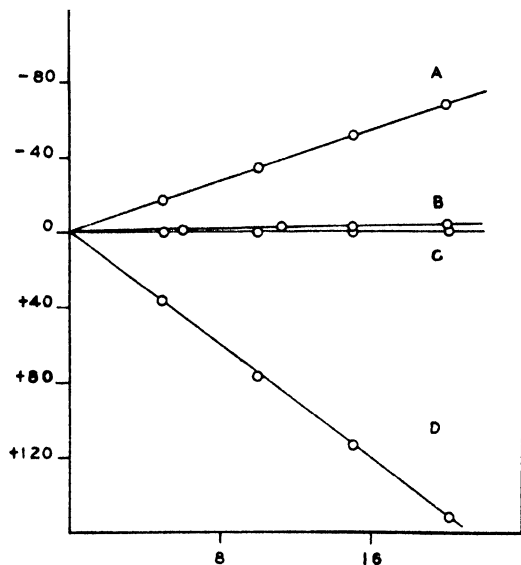


FIG. 1. Effect of carotene and of oxidized carotene on the oxygen uptake of linoleic acid. Curve C represents the oxygen uptake of 2 mg. of carotene; Curve B that of 2 mg. of oxidized carotene. Curve A represents the *excess* oxygen consumption of a mixture of 0.5 cc. of linoleic acid plus 2 mg. of oxidized carotene over that of 0.5 cc. of linoleic acid alone; Curve D, the decrease in oxygen consumption of a mixture of 0.5 cc. of linoleic acid plus 2 mg. of carotene as compared with that of 0.5 cc. of linoleic acid alone (see text). The ordinates represent oxygen uptake in c.mm.; the abscissæ, time in minutes.

*Effect of Vitamin A on Oxidation of Linoleic Acid*—Since it has now been clearly demonstrated that carotene may be transformed into vitamin A in the animal organism we determined to test the effect of this vitamin on the oxidation of unsaturated fatty acids. The unsaponifiable material of cod liver oil was the source of

vitamin A in these experiments. This material was extracted with ether and the ether evaporated; the residue then contains most of the vitamin A present in the original oil. It was found necessary to prepare the vitamin extract fresh before each experiment since vitamin A, like carotene, becomes oxidized if exposed to air for any length of time or if dissolved in an autoxidizable acid such as linoleic acid.

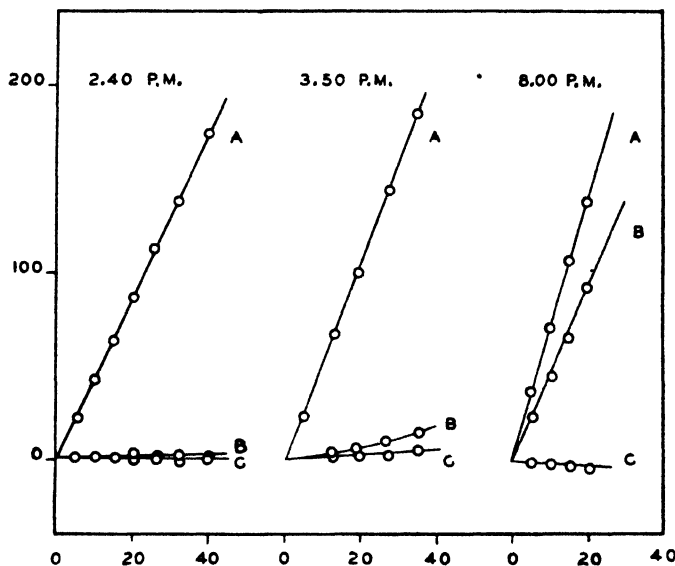


FIG. 2. Effect of vitamin A on the oxygen uptake of linoleic acid. Curve A is for 0.5 cc. of linoleic acid; Curves B and C, the same, plus unsaponifiable matter from cod liver oil (Curve B is for one-tenth as much of the cod liver oil extract as is Curve C). The ordinates represent oxygen uptake in c.mm.; the abscissæ, time in minutes.

It was found that vitamin A affects the oxidation of linoleic acid qualitatively the same as carotene. Fig. 2 shows the effect of two concentrations of the vitamin. Curve A represents the oxygen uptake of linoleic acid alone; Curves B and C represent the uptake of the same amount of linoleic acid plus two concentrations of the vitamin preparation. In experiments of this kind it is essential that the vitamin preparation be mixed with the linoleic acid and measurements started as quickly as possible to



avoid partial oxidation of the vitamin. Hence in this experiment instead of dissolving weighed quantities of the extract in the acid, a solution of empirical strength was made in linoleic acid, the concentration in Curve B then being adjusted to one-tenth that in Curve C by dilution. It will be observed that both concentrations

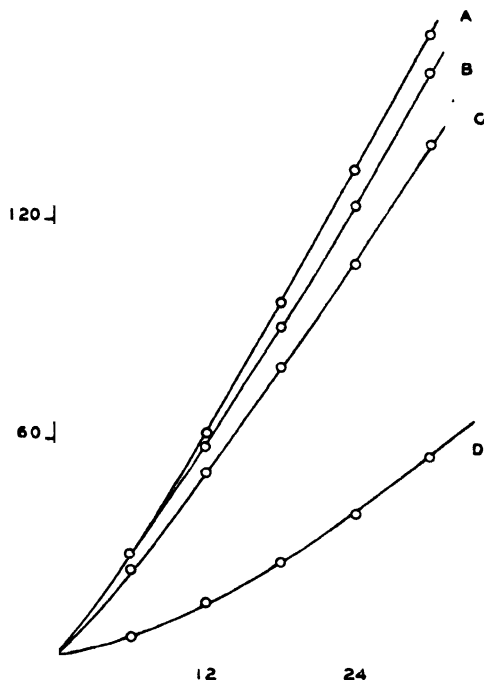


FIG. 3. Effect of low concentrations of vitamin A on the oxygen uptake of linoleic acid. Curve C is for 1 cc. of linoleic acid; Curves A, B, and D, the same plus 0.05 mg., 0.5 mg., and 5 mg. respectively of the unsaponifiable matter from cod liver oil. The ordinates represent oxygen uptake in c.mm.; the abscissæ, time in minutes.

of the unsaponifiable matter inhibited completely the oxygen uptake of linoleic acid during the 1st hour. During the 2nd hour the less concentrated of the two vitamin solutions was starting to take up oxygen at a slow rate. In the 6th hour the linoleic acid containing the less concentrated vitamin was being oxidized at a fairly rapid rate while the more concentrated preparation was still completely inhibited. Similar results were obtained in five experi-

ments. Substitution of an ether extract of rat liver for the cod liver oil preparation also gave similar results.

In order to secure complete inhibition for a measurable time it was found necessary to start with concentrations of 2 mg. or more of cod liver oil extract. This is probably equivalent to less than 0.02 mg. of vitamin A, for Drummond and Baker (9) estimate that vitamin A makes up less than 1 per cent of the unsaponifiable material of cod liver oil. When very small amounts of the extract (less than 1 mg.) were weighed out and dissolved in linoleic acid, all of the vitamin might have become oxidized in the 1st half hour or so required to set up the experiment, before readings could be made, so that a slight acceleration rather than an inhibition might be obtained (see Fig. 3).

In order to demonstrate that it was actually the vitamin in the cod liver oil extract that was responsible for the observed effects, the antimony trichloride test for vitamin A was employed. For references see the recent work of Brode and Magill (10). The extracted material was allowed to remain in contact with air for several days until the vitamin was entirely oxidized, as shown by the negative reaction to the antimony trichloride test. Upon dissolving such material in linoleic acid and measuring the rate of oxidation, it was found in three experiments that no inhibition of oxidation could be observed; indeed, like oxidized carotene, the substance produced only an acceleration of oxidation. There remains, of course, the possibility that these effects are due to the presence of some antioxidant other than the vitamin, but associated with it and subject to oxidation in a similar manner. This possibility has not been disposed of as yet.

*Effect of Hemoglobin on Vitamin A*—Since it is now well known (Robinson (11), Kuhn and Meyer (12)) that hemin compounds act as catalysts in the oxidation of unsaturated fatty acids, we performed a few experiments to test the effect of vitamin A on the oxygen uptake of linoleic acid in the presence of hemoglobin. It was found that the vitamin was quickly destroyed under these conditions with the resulting acceleration in rate of oxygen consumption of the linoleic acid which was greater than the acceleration caused by the addition of hemoglobin alone. These results are in line with the observations of Jones (13), McCollum, Simmonds, and Becker (14), Taylor and Nelson (15), and others which show that iron salts tend to destroy vitamin A.

## DISCUSSION

Since vitamin A prevents the oxidation of unsaturated fatty acids *in vitro* it seems logical to seek a functional relationship in the animal body between vitamin A and fat metabolism. And since linoleic acid and other more highly unsaturated fatty acids are present in the body most constantly in the form of phospholipids, the possibility of an association between vitamin A and phospholipid metabolism suggests itself. The hypothesis of Leathes and Raper (16) that the liver is the seat of phospholipid formation adds weight to the above suggested relationship, for Moore (17) has demonstrated that most of the vitamin A in the body is concentrated in the liver.

The pathology of vitamin A deficiency also supports the above suggested relationship. The cessation of growth typical of vitamin A deficiency is accompanied by specific lesions of glandular tissues which contain relatively large amounts of phospholipids of a high degree of unsaturation. Cytological evidence points to the conversion of the cells of secreting epithelia to a keratinized, inactive type of cell. Such changes must involve deep seated modification of cellular elements such as the Golgi apparatus and mitochondria which are phospholipid in nature. Experiments designed to test this suggested importance of vitamin A as a regulator of phospholipid metabolism in the animal organism are well under way. Preliminary results indicate a large difference in the phospholipid content of normal and of vitamin A-deficient rats. Experimental data covering this work will be communicated at an early date.

## SUMMARY

1. Carotene, the precursor of vitamin A in the animal body, greatly inhibits the oxygen uptake of linoleic acid. Oxidized carotene, on the other hand, slightly accelerates the oxygen uptake of this acid.
2. Vitamin A in small concentrations may completely inhibit the oxygen uptake of linoleic acid for some hours. This inhibition wears off, as in the case of carotene, when the vitamin is destroyed by oxidation.
3. The possibility that vitamin A may be concerned with phospholipid metabolism is discussed.

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## THE ALLEGED INCREASE IN PLASMA FATS AFTER THE INJECTION OF EPINEPHRINE

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Himwich and Spiers (1) have recently published experiments which they claim show an increase in plasma fats following the injection of epinephrine. Similar findings are reported after emotional excitement in cats (Himwich and Fulton (2)) and after nocuous stimulation in cats (Fazikas, Spiers, and Himwich (3)). Finally, Koskoff and Dusser de Barenne (4) have claimed that stimulation of the central end of the sciatic nerve leads to a rapid increase in plasma fats.

It is now well known that all the above procedures also increase the blood sugar through the liberation of epinephrine, so presumably the results observed by the above workers are all due to this substance. On examination of their data one is at once struck by the close parallelism between the changes in blood sugar and the so called plasma fats.

Previous to the work of Himwich and Spiers (1) Rony and Ching (5) had reported experiments in which they injected similar amounts of epinephrine into dogs and found no significant changes in plasma fats.

The chief difference in the experiments of these two groups is the method employed for the estimation of the plasma fats. Himwich and his coworkers used a method (6) which in its essentials is but little different from that of Stewart and White (7). The principle of this method is as follows:

The plasma is run into an alcohol-ether mixture (3:1), heated to boiling, and the precipitated proteins are filtered off. The filtrate is then made up to known volume with more alcohol-ether mixture and 20 cc. of this (representing 1 cc. of the original plasma)

are saponified by heating for 2 hours on a steam bath with *exactly* 5 cc. of 0.1 N NaOH. 5 cc. of *exactly* 0.1 N HCl are then added and the mixture boiled down to 1 cc. Absolute alcohol is then added, the mixture heated to boiling, and titrated with 0.1 N or 0.05 N NaOH, phenolphthalein being used as indicator.

The chief point to be observed about the above method is that the fatty acids are not separated from the saponification mixture before titration and it is therefore easily understood that if any acids were formed from other substances in the plasma extract as a result of the saponification they would be included in the final titration and estimated as fatty acids.

Rony and Ching used Bloor's new oxidative method (8) to determine fatty acids. In this method the fatty acids are extracted from the saponification mixture with petroleum ether before estimation and, although this method might also include any other acids not arising from the plasma fats, the quantity extracted would probably be much less.

In the work reported here we have always estimated the plasma fatty acids by a third method, in addition to the Stewart and White method. This method was originally introduced by Stoddard and Drury (9) and with slight modifications has since been adopted by Stewart (*cf.* Stewart, Gaddie, and Dunlop (10)). In this last method, after saponification of the plasma extract, the fatty acids are precipitated with *excess* of HCl, filtered off either through a Gooch crucible or a filter paper, and washed with 5 per cent NaCl. They are then dissolved in hot alcohol and titrated.

As stated above, the original Stewart and White technique would include in the final titration any other acids formed during the saponification as well as the fatty acids. Stewart, Gaddie, and Dunlop state that they recognize that the original method would include the phosphoric acid liberated by the hydrolysis of the phospholipids, although this source of error is still perpetuated in the modified method of Himwich, Friedman, and Spiers (6).

There is, however, a more serious objection to the Stewart and White method which is also true for that used by Himwich and his coworkers. It is a well established fact that under the influence of alkali glucose is broken down into a complex mixture of organic acids, and indeed this reaction forms the basis of Moore's test for reducing sugars. An examination of the flasks during the

saponification of plasma extracts shows in all cases the development of a marked brown color which increases with the sugar content of the plasma under investigation. It is obvious that if water-soluble organic acids are produced from glucose during saponification they would be titrated as fatty acids in all methods in which the fatty acids are not precipitated out or otherwise separated from them before estimation. If such an acid breakdown is really occurring in the glucose present in alcohol-ether extracts of plasma when analyzed for fatty acids by the Stewart and White method, then the results obtained by Himwich *et al.* are capable of another interpretation. All the above procedures such as injection of epinephrine, nocuous stimulation, etc., are well known to lead to marked increases in blood glucose. These increases in blood glucose would appear in the Stewart and White method as increases in fatty acid and would thus account for the results obtained. That this is actually the case is in our opinion borne out by the following experiments.

*Experiment 1*—The alcohol-ether extract of plasma contains reducing substances (*cf.* Bloor (8)). This is shown by the fact that after evaporation to dryness, extraction with water, and filtering, the filtrates obtained strongly reduce Somogyi's sugar reagent giving amounts of glucose comparable to those originally present in the plasma. Furthermore it can be shown that after saponification with NaOH this reducing value has disappeared while there have appeared amounts of lactic acid which increase with the sugar content of the plasma.<sup>1</sup>

Glucose content per 100 cc. plasma			Lactic acid content, per 100 cc. plasma, after saponification
Original plasma	Plasma extract	After saponification	
<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
123	118	16	157
288	223	18	
343	335	18	246
405		18	352

<sup>1</sup> It is of course realized that the alkaline degradation of glucose yields other acids besides lactic acid. Some of these might be estimated as lactic acid by the Friedemann, Shaffer, and Cotonio method. Any remaining glucose was removed with copper sulfate and lime before the determination of the lactic acid.



*Experiment 2*—We have also reinvestigated the changes in the anesthetized cat of the blood sugar and plasma fats during the continuous infusion of epinephrine. The blood sugar was estimated by Folin's method (11) and the plasma fats by the original Stewart and White method as well as by the Stoddard and Drury method.

A cat weighing 2.86 kilos was anesthetized with dial. 3.4 mg. of epinephrine were injected into the jugular vein at the rate of 1.7 mg. per hour. Blood samples were withdrawn before and at intervals during the injection from a carotid artery.

Time	Blood sugar per 100 cc.	Plasma fats, per 100 cc., as tripalmitin	
		Stewart and White method	Stoddard and Drury method
	gm.	gm.	gm.
Before epinephrine.....	0.161	0.860	0.323
After perfusion for $\frac{1}{2}$ hr.....	0.313	1.775	0.323
“ “ “ 1 “.....	0.324	1.785	0.323
“ “ “ $1\frac{1}{2}$ hrs.....	0.324	1.720	0.296
“ “ “ 2 “.....	0.324	1.745	0.296

This experiment shows (a) the close parallelism between the blood sugar and the plasma fats as estimated by Stewart and White's method, (b) the absence of any effect of epinephrine on the plasma fats as estimated by Stoddard and Drury's method, thus confirming the findings of Rony and Ching (5), (c) the marked difference in the resting value for plasma fats by the two methods. As was pointed out by Stewart, Gaddie, and Dunlop (10), part of this at least is due to the inclusion in the Stewart and White method of the phosphoric acid liberated from the phospholipids. In addition it is our contention that a further part is due to the formation of organic acids from glucose during the saponification.

*Experiment 3*—A cat weighing 4.25 kilos was anesthetized with dial and eviscerated; but the kidneys and adrenals were left intact. A 25 per cent glucose solution was infused at the rate of 13 cc. an hour. 20 units of insulin were given intravenously at the start of the infusion and repeated every hour. Blood sugar and plasma fats were estimated by the same methods as in Experiment 2.

Time	Blood sugar per 100 cc.	Plasma fats, per 100 cc., as tripalmitin	
		Stewart and White method	Stoddard and Drury method
	gm.	gm.	gm.
Before infusion.....	0.181	0.605	0.309
After 1 hr. infusion.....	0.449	1.530	0.255
“ 2 hrs. infusion.....	0.495	1.770	0.215
“ 3 “ “ .....	0.607	2.120	0.202
$\frac{1}{2}$ hr. after stopping infusion.....	0.432	1.585	0.202

The metabolism of the eviscerated cat amply supplied with glucose and insulin is almost entirely maintained at the expense of carbohydrate (Best, Dale, Hoet, and Marks (12)), yet the plasma fats as estimated by Stewart and White's method steadily increase. This is obviously impossible since the metabolism is exclusively of the carbohydrate type and since, in addition, the liver is not functioning. The plasma fats as estimated by Stoddard and Drury's method show a decrease during the period of infusion which we believe is a much more reasonable finding.

The claim of Koskoff and Dusser de Barenne (4) that a reflex hyperlipemia follows the stimulation of the central end of a sciatic nerve in the anesthetized cat can also be shown to be due to the same cause.

*Experiment 4*—A cat weighing 2.95 kilos was anesthetized with dial. A blood sample was withdrawn from a carotid artery. Immediately following this the intact left sciatic nerve was periodically stimulated with 5 second tetani, allowing 5 seconds

Time	Blood sugar per 100 cc.	Plasma fats, per 100 cc., as tripalmitin	
		Stewart and White method	Stoddard and Drury method
	gm.	gm.	gm.
Before first stimulation.....	0.128	0.511	0.242
Immediately after 5 min. inter- mittent tetanus.....	0.174	0.900	0.242
Before second stimulation $1\frac{1}{2}$ hrs. later.....	0.155	0.645	0.228
Immediately after 10 min. inter- mittent tetanus.....	0.200	1.020	0.202

between each. This was kept up for 5 minutes. At the end of the stimulation another blood sample was withdrawn. Blood sugar and plasma fats were estimated as before. After  $1\frac{1}{2}$  hours of rest the above procedure was repeated except that the stimulation was continued for 10 minutes. The secondary of the induction coil was at 10 cm. in both cases.

The final proof of the effect of glucose on the estimation of plasma fats by Stewart and White's method is shown in the following experiment.

*Experiment 5*—A sample of plasma was taken and its glucose and fat content estimated as before. To different portions of this plasma increasing amounts of glucose were added and the sugar and fat content again estimated in each specimen.

	Glucose content per 100 cc.	Apparent plasma fat con- tent per 100 cc.  Stewart and White method
	gm.	gm.
Plasma alone.....	0.181	0.751
" + glucose.....	0.362	1.398
" + ".....	0.543	1.960
" + ".....	0.724	3.370
" + ".....	0.905	4.540

This experiment clearly shows that even *in vitro* as the glucose content of the blood increases so does the apparent plasma fat, as estimated by Stewart and White's method. The plasma fats estimated by Stoddard and Drury's method did not change.

In conclusion it can be stated that the original Stewart and White method for the estimation of plasma fat contains two sources of error: (a) the phosphoric acid liberated from the hydrolysis of phospholipids is titrated as "fatty acid;" (b) under the influence of alkali the glucose present in the plasma extracts is converted into a mixture of organic acids which are also included as "fatty acids." For these reasons the fatty acid content of any plasma will be too high if estimated by this method. In addition, although there will be a good recovery of added fatty acids by this method (so long as the glucose content of the plasma remains constant) any changes in blood glucose will lead to error, since the increase or

decrease in glucose will be followed by an apparent parallel change in plasma fatty acids. This in some cases will entirely mask the true physiological changes.

The results of Himwich *et al.* and Koskoff and Dusser de Barenne are all open to criticism on these grounds since the method they have employed contains both the sources of error mentioned above. We have repeated certain of their experiments, chiefly those in which agencies were employed which are well known to increase blood sugar and in every case as measured by their method, the plasma "fatty acids" also increased, although the estimation of these substances by a method which does not include the errors mentioned above showed this conclusion to be erroneous. Finally, the fact that the mere addition of glucose to plasma increases the apparent fat content would seem to necessitate a revision of all data that have been collected by the use of these methods, that is, those of Stewart and White (7), and Himwich, Friedman, and Spiers (6).

For example, Hill, Long, and Slight (13) have recently used the Stewart and White method to study the changes in plasma fats in certain cases of mental depression. It is now realized that the total values are too high for the reasons given above but in these cases the blood sugar changes were so slight that they would not greatly affect the conclusions already drawn from the observed increases after a fat meal. We are, however, repeating this work using a more suitable method, and until such time as this is completed the previous results are to be regarded as *sub judice*.

#### SUMMARY

1. Owing to the fact that during saponification of alcohol-ether extracts of plasma the glucose present breaks down to organic acids, the methods of Stewart and White and of Himwich, Friedman, and Spiers are of no value for the estimation of the true plasma fatty acids. A second source of error in these methods is the inclusion by them as "fatty acids" of the phosphoric acid liberated by the hydrolysis of phospholipids.

2. The results obtained by certain workers on the increase in plasma fatty acids after the injection of epinephrine, nocuous stimulation, fright, etc., are in all cases to be largely attributed to the concomitant increases in blood sugar. Analyses of the plasma

by a method free from the above errors leads to different conclusions in all the cases we have examined.

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## THE STIMULATION OF YEAST GROWTH BY THALLIUM, A "BIOS" IMPURITY OF ASPARAGINE

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Different brands of asparagine give different yields of yeast when all other conditions are identical (Richards, 1925). Asparagine is a good source of nitrogen in organic combination and has been used in culture media for unicellular organisms for years (Stern, 1899). With the exception of the different yields obtained with different brands of asparagine, Williams' medium (1920)<sup>1</sup> was found by the writer to be very satisfactory for the growth of the pure strain of *Saccharomyces cerevisiae* Hansen derived from an isolated, single cell.

The changes which take place in the medium with the growth of yeast have been measured and described in detail elsewhere (Richards, 1931, 1932, *a*). A few cells seeded into 10 cc. of the medium in a Pyrex test-tube grow at a constant rate until the excretion products of fermentation accumulate in the medium to a threshold concentration. The rate of growth of the population then decreases as part of the larger buds are selectively killed and as the food is utilized. The result of these effects is an equilibrium number of the population. This marks the end of the first cycle of growth.

Recently Williams *et al.* (1927) reexamined their medium and found it satisfactory for the growth of yeast. I have used a series of concentrations of asparagine and found that the amount in Williams' formula was optimal.

The materials in the medium, exclusive of the asparagine and sugar, are greater than the amount needed by the yeast, as may be

<sup>1</sup> Sucrose 20 gm.,  $(\text{NH}_4)_2\text{SO}_4$  3 gm.,  $\text{KH}_2\text{PO}_4$  2 gm., asparagine 1.5 gm.,  $\text{CaCl}_2$  0.25 gm.,  $\text{MgSO}_4$  0.25 gm., distilled  $\text{H}_2\text{O}$  1000 cc.

shown by following the qualitative analysis schemes in which the volumes are kept strictly comparable and the density of the different precipitates is compared. Comparing the cell-free medium with fresh medium shows that even after the yeast has grown in the medium for 700 hours the  $\text{SO}_4$ ,  $\text{PO}_4$ ,  $\text{NH}_4$ , and Ca content of both is essentially the same. The Cl and Mg are reduced to approximately one-half as much as in the fresh medium. It is unlikely, therefore, that these substances limit the growth of the yeast.

The extent of the differences of yield for the different brands of asparagine is shown in Fig. 1. The equilibrium yield at the end of the first cycle of growth of the yeast population with Eimer and Amend 1929 asparagine is 63 per cent of that obtained when Merck asparagine is used. In these experiments the medium was made up without the asparagine and separate amounts were placed in separate beakers, then a weighed amount of each asparagine was added. All batches were treated otherwise exactly the same. The curves in Fig. 1 are averages of two independent tests. Each series contained three separate tubes of medium with each asparagine.

During the summer of 1929 an opportunity occurred for the investigation of the differences between different asparagines. 50 gm. lots of the five asparagines shown in Fig. 1 were obtained and an attempt to discover the difference was made. The moisture, ash, total nitrogen, formol titration, and optical rotation<sup>2</sup> were determined. All of the asparagines were imported, as shown in Table I. The old Eimer and Amend 1911 sample had been on the shelves of the Marine Biological Laboratory chemical room since that date. It was too old to try to trace its source and the lot number was no longer legible. Later similar analyses of some samples prepared by the Difco Laboratories in this country were made and they are included in Table I. The growth yield of these was obtained with reference to a control containing Merck asparagine.

The moistures were nearly the same and a little less than the theoretical amount of 13.6 per cent for 1 HOH of crystallization.

<sup>2</sup> I wish to express my appreciation to Mr. W. A. Wolff, of the Department of Physiological Chemistry, University of Pennsylvania, for making the optical rotation measurements.

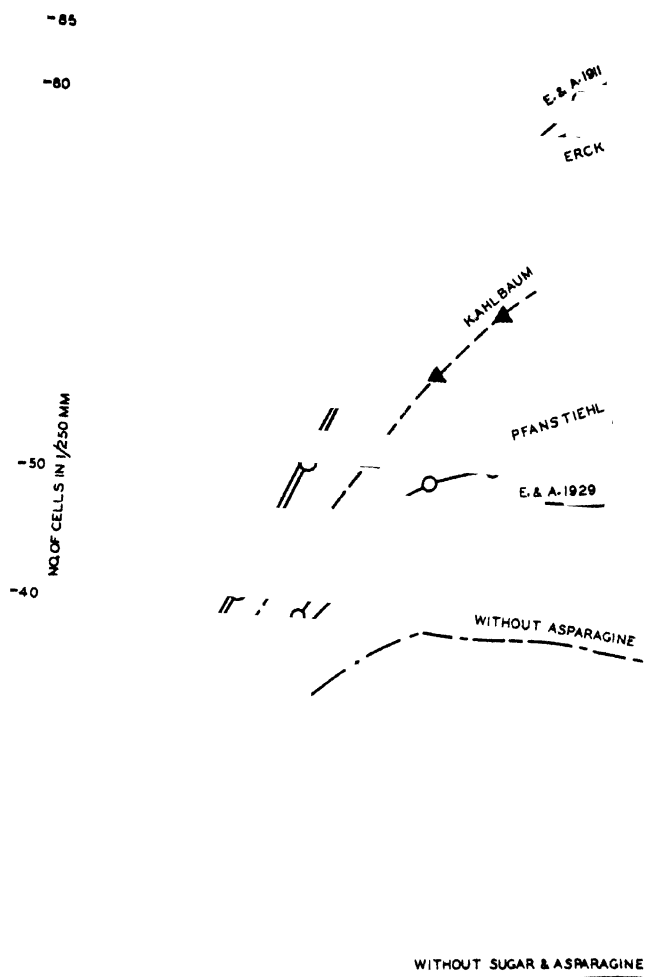


FIG. 1. The growth of yeast in Williams' medium containing different brands of asparagine, without asparagine, and without asparagine and sugar. (E. and A. = Eimer and Amend.)



TABLE I  
*Analyses of Commercial Samples of Asparagine*

	Eimer and Amend		Merck	Kahlbaum	Pfanstiehl	Difco		
	1911	1929 J79				Stock	Special	
							1929 8917	1930 21670
Date.....			1929 21769	1929 1G112T	1929 5732			
Lot No.....								
Source.....		Theodor Schuch- ardt, Götting	E. Merck, Darmstadt	Schering- Kahlbaum, Berlin	Imported	Prepared at Detroit		
Moisture, per cent.....	12.0	12.0	11.9	11.9	13.0	11.8	12.9	13.9
Ash, per cent.....	0.001	0.001	0.023	0.001	0.024	0.02*	0.02*	0.002*
Kjeldahl N†.....	0.517	0.482	0.434	0.510	0.448	0.443	0.427	0.424
Formol titration‡.....	19.30	19.34	19.41	18.99	19.17	19.33	17.58	19.13
Optical rotation, degrees§.....	25.2	26.5	26.0	25.4	25.2			
pH stock solution.....	4.3	4.6	4.3	4.4	4.5	4.4	4.6	4.6
Yield of yeast, per cent.....	105	63	100	84	77	78	77	69

\* Made with 1 gm., so not directly comparable to the other determinations made with 5 gm.

† Expressed as mg. of NH<sub>4</sub> per 1.5 mg. of asparagine (*cf.* text).

‡ Expressed as cc. of 0.01 N NaOH to neutralize (Northrop, 1926).

§ Asparagine dissolved in 0.4 N HCl.

They were dried in an oven at  $105^{\circ}$  to a constant weight within 24 hours. The ash content varied. The Kjeldahl nitrogen is expressed as mg. of  $\text{NH}_3$  per 1.5 mg. of asparagine which are contained in 1 cc. of the medium. All samples contained more than the 0.386 mg. theoretically present. The formol titrations did not show significantly different hydrolysis in the different samples. The optical rotations made with the asparagine dissolved in 0.4 N HCl are nearly identical. While small differences are shown between the different brands none of these differences correlates directly with the difference in yield of yeast. Consequently the difference in the growth of yeast with these must be due to some impurity present in the asparagine rather than to the asparagine itself.

The identification of Bios I as inactive inosite by Miss Eastcott (1928) suggested that this substance might be present. It was found to increase the growth of the yeast (Richards, 1932, *b*) when it was added to the culture medium. Adding inosite to the Eimer and Amend asparagine, which alone gave less growth, resulted in a greater increase than was found from adding inosite to the richer Merck asparagine. While there are several color tests for inosite, none of them is very satisfactory (Needham, 1926) and no positive tests were found.

I am indebted to Dr. R. J. Anderson for testing the Merck asparagine for inosite. He found no inosite present although his method was reliable for less inosite than would have been expected had the differences in yield of yeast been due to this substance.

The next step toward the understanding of the impurity was made possible by the Difco Laboratories through the courtesy by Mr. H. G. Dunham. When they were preparing asparagine he had saved for me samples from the first crystallization through and including the sixth recrystallization—three more recrystallizations than are usually made with the commercial product. The differences in yield of these asparagines are shown objectively in Fig. 2. Much of the material which is associated with increased growth of yeast is lost from the second recrystallization. The third and fourth purify it more, but there is little difference between the products from the fifth and sixth recrystallizations. For a basis of comparison a Merck sample of asparagine was also used under identical conditions. These results also indicate the pres-

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ence of an impurity present in commercial asparagines which increases the growth of yeast.

The Difco Laboratories then prepared a desiccated sample of the liquors from the crystallizations of a preparation of asparagine.

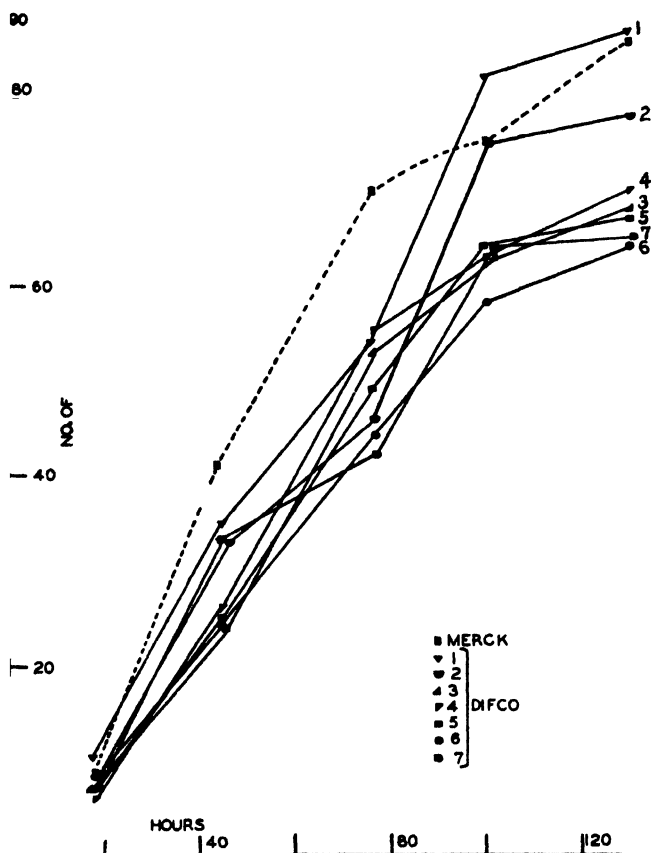


FIG. 2. The growth of yeast in medium containing Merck asparagine and Difco Laboratories asparagine (marked Difco). Curve 1 is the asparagine of the first crystallization, Curve 2 of the first recrystallization, Curve 3 of the second recrystallization, etc. Arithlog plot.

The material readily dissolves in water and forms a reddish brown, bitter, astringent solution. The addition of 0.01 mg. per cc. of this material increases the growth of yeast beyond that of the

control (Fig. 3). The addition of 0.1 mg. gave greater growth and 1 mg. distinctly more growth. The medium of these experiments was the usual formula with Merck asparagine. A stock solution was made containing 200 mg. in 20 cc. and suitable portions added

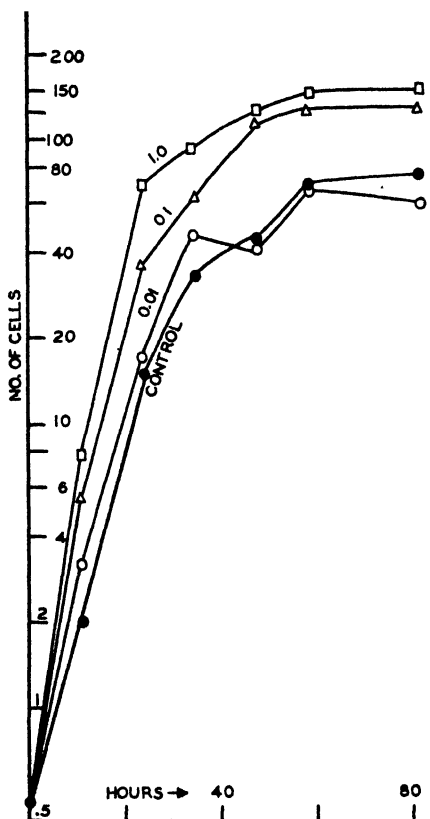


FIG. 3. The growth of yeast when varying amounts (mg. per cc.) of the desiccated mother liquors from which the asparagine is crystallized during its preparation are added to the culture medium. Arithlog plot.

to the medium. Treating this with norit "A" (Eimer and Amend) and filtering changed the color to a very pale yellow. The addition of a volume of this equal to 0.1 mg. per cc. of the original liquid gave less growth than did the 0.1 mg. of the untreated solution for the first cycle of the growth, indicating the removal

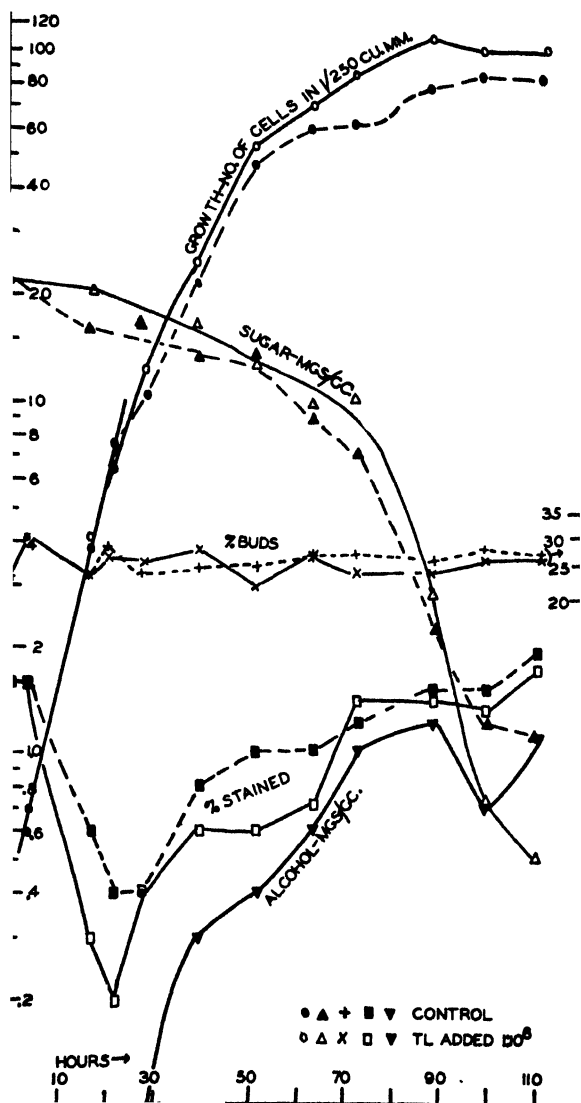


FIG. 4. The analysis of the increased growth obtained with the addition of thallium to the medium (1Tl:10<sup>8</sup> medium). Cf. text. The dark symbols and discontinuous line represent the control cultures and the open symbols and the continuous line represent the cultures containing thallium.

of some of the stimulating material. This sample of desiccated mother liquors included the first extraction liquid and contained so many substances that it seemed hopeless to try to determine its constituents.

During a discussion of these problems with Professor L. B. Mendel of Yale University, he made the helpful suggestion that I ash some of the asparagine to determine whether the impurity was organic or inorganic. Some Merck asparagine was carefully ashed in a platinum crucible heated not more than to a dull red over an alcohol flame. The addition of 0.003 mg. of the ash per cc. of medium containing Eimer and Amend asparagine gave an increased yield of 24 per cent more than that of the control. This demonstrated that the impurity was *inorganic* rather than organic.

The next time the Difco Laboratories prepared asparagine they saved separately and sent to me the desiccated mother liquors of the first, second, and third recrystallizations. The yield when 0.05 mg. per cc. of these was added to the medium with Eimer and Amend asparagine was respectively 195, 186, and 153 per cent of the yield of the control cultures.

A 10 gm. sample of the mother liquor of the second recrystallization was carefully ashed, but even after 3 days heating at a dull red it still contained considerable carbon. This residue was extracted with a few cc. of boiling distilled water and the extract was tested with the micro methods of Chamot and Mason (1931). The only unusual constituent found was thallium.

Some thallium was obtained and a concentrated solution was made of the acetate, as it was the most soluble thallium salt available. The addition of thallium to the medium gave the following results.

	Asparagine								
	Merck	Difco	Eimer and Amend						
TlAc added, mg. per cc.....	None	None	None	10	1	0.1	0.01	0.001	0.0001
Yield, per cent.....	168	141	100	1.5	4	5	144	159	125

Greater concentrations than 0.01 mg. per cc. were toxic and less than 0.001 mg. per cc. was suboptimal. When thallium was added to the medium containing Merck asparagine the optimal amount

was slightly less, being 0.00014 mg. per cc., and the yield was 124 per cent of the control. Two other series of experiments with medium with Eimer and Amend asparagine indicated that the optimal concentration was 0.001 mg. of thallium per cc. with thallium chloride, and the yields were 176 and 181 per cent of that of the control cultures. Proportionate amounts of the sulfate also gave similar results. All of these experiments indicate that thallium is a growth stimulant for this species of yeast when it is added to Williams' medium. Thallium has been reported to stimulate the growth of yeast by Gottbrecht (1880) and by Schulz (1886).

The amounts of ash obtained from the available amounts of asparagine were too small to give conclusive tests with the micro methods of Chamot. In order to obtain a more critical test the spectroscopic method of Ramage (1929) was used. The ash was wrapped in Whatman No. 40 filter paper and burned in a gas and oxygen flame and the spectra photographed with a quartz Hilger spectroscope.<sup>3</sup> Tests with known thallium salts showed that distinctly less than 1 mg. could be detected in this manner. The 5351 and 3776 lines were found in the photographs of the spectra from Merck and Difco asparagines and in the ash of the second recrystallization mother liquor. With the latter the 3230 line was also observed. The lines were faint but identified by measurement and by direct superimposition with a spectrum of known thallium. This demonstration of the presence of thallium in the ash of the asparagine and of the mother liquor from which it is crystallized, together with the fact that very small amounts of thallium do stimulate the growth of yeast, shows that thallium is the impurity which caused the different yields obtained with the different brands of asparagine. Recrystallization produced a purer asparagine with little of this impurity which contributes to the growth of yeast.

The increased growth of a population of yeast obtained when 1 part of thallium is added to 10<sup>6</sup> parts of medium containing Eimer and Amend asparagine is due to less selective killing of the cells and to a less rapid removal of sugar from the culture medium (Fig. 4). The injured or killed cells stain with dilute methylene blue, which

<sup>3</sup> The Physics Department of Yale University made this possible by furnishing the instrument, and I am indebted to Professor W. W. Watson for his helpful interest in the determination.

gives an index of the death rate. The alcohol production of the cultures is about the same, but the decrease in budding (birth rate) occurs later in the cultures containing thallium. In Fig. 4 the growth rates are very nearly the same but in two other series of experiments the rate of growth (ratio of the tangents during the period of logarithmic growth) of the thallium-containing cultures was 108 and 114 per cent greater than that of the control cultures. For details of the analytical methods see Richards (1932, *a*).

Since Wildiers' (1901) report of an unknown substance which was essential for the growth of yeast, many attempts have been made to isolate and identify this "bios" material as Wildiers provisionally named his hypothetical substance. Wildiers concluded that this unknown substance was organic because he did not get increased growth by the addition of yeast ash to his medium. If thallium was present in the ash, and he added amounts that would give concentrations of thallium distinctly above or below the optimum concentration, no increased growth would have occurred. As he did not give the amounts of ash added, no certain conclusion is now possible. Wildiers found that the addition of asparagine to the medium did not give the increased growth that extracts containing the unknown substance gave. Had he used a relatively pure asparagine similar to the Eimer and Amend product the additional growth would not have occurred. Consequently, it is believed that the experiments of Wildiers did not exclude the possibility that thallium might have been the unknown growth stimulant.

Darby (1930) has suggested that the poor growth obtained with some of the media used in Wildiers' laboratory is due to the media's being too alkaline. The reason for Darby's results has been given by Elvehjem (1931) to be that the iron and copper are precipitated out of the medium as phosphates when it is too alkaline. Without these elements the yeast growth is very poor. The addition of 0.001 mg. per cc. of iron to medium containing etiolated yeast gave an increase in growth of nearly 400 per cent. Elvehjem found also that the addition of the same amount of copper increased the yield further but proportionally less. The inorganic salts and sugar of reagent grade (Merck's Blue Label) used in my experiments contain enough iron and copper, as impurities, because on addition of 0.001 mg. of iron per cc. and of copper in the form of chlorides there



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was no increase with the added iron and slightly less growth when the copper was added.

Different groups of investigators have found various substances which stimulate the growth of yeast. The earlier literature has been reviewed by Tanner (1925). Because of Wildiers' conclusion that the unknown substance that he endeavored to find was of organic nature much of the modern work has neglected the inorganic requirements of the yeast. The several organic substances which have been isolated have given widely different results with different strains of yeast, as may be seen by consulting the papers of Miller (1930), Williams and his students, and Richards (1932, *b*). Tanner *et al.* (1926) conclude that different yeasts have different dietary requirements after studying some 50 species of yeast. The latter conclusion is supported by my experiments.

Certain of the substances investigated can be removed by adsorption methods. Thallium, and probably other heavy metals, can be adsorbed by activated charcoal such as norit "A." Treating a solution of thallium with one-tenth of its volume of norit for 10 minutes removed enough thallium to make a difference of 15 per cent in the yield of the yeast.

Williams and his associates have used his medium in their studies of growth stimulants for different yeasts. It is probable that part of the differences which they find with different products and different strains of yeast are due to the fact that the medium is not equally suitable for the several yeasts used. Different brands of asparagine will affect their results, as shown in this paper.<sup>4</sup> This possible variation of their medium seems not to have been considered by them beyond the statement that "We have also observed the occurrence of 'bios' in highly purified asparagine and Kahlbaum's lactose" (Williams, Warner, and Roehm, 1929, p. 2772). It is believed that an inorganic element like thallium would be separated by the method of fractional electrolysis and that, because there is an optimal concentration for growth and because decreased growth is associated with greater and lesser concentrations, this element may explain some of the observations of Williams and Truesdail (1931). They should determine

<sup>4</sup> Williams has used Yeast 2335 from the "American type culture collection." My yeast is a pure strain from a single cell isolated from this same yeast (*cf.* Richards, 1932, *a*, *b*).

whether the stimulants in their extracts are organic or inorganic. Until more is known of the nutrition of yeast, especially of the inorganic impurities of the constituents of the media employed, it would seem premature to speculate on the relation of yeast nutrients to vitamins as Williams has done since his early attempt to identify bios with vitamin B (Williams (1920) and subsequent papers). If Williams' medium contained *only* the chemicals named in the formula, it is doubtful whether the yeast could grow in it. (Cf., *e.g.*, the need of iron mentioned before, Elvehjem, 1931.)

The writer believes that attempts to isolate and identify complex organic materials alleged to be essential for the growth of yeast will not be successful until the inorganic constituents of culture media for yeast are known to contain all of the necessary materials for the nutrition of the species of yeast to be studied. It is quite possible that the various bioses which have been discovered and their fractions may contain essential inorganic nutrients in the same way that certain brands of asparagine were found to contain thallium. Additional study will be necessary to make certain that thallium is an essential food for yeast, but it does increase the growth of the strain of yeast used in my experiments and has been reported to increase yeast growth by other investigators during the nineteenth century. Further investigation of the inorganic nutrition of yeast is under way and will be reported in the future.

#### SUMMARY

When different brands of asparagine are used in Williams' culture medium, the yield of yeast obtained, conditions otherwise being constant, may differ by 65 per cent. This difference is shown to be due to thallium, an impurity in certain of the asparagines which was identified spectroscopically. Asparagine which is further purified by recrystallization gives less growth of yeast. When the asparagine sold by Eimer and Amend is used in the medium the addition of 0.001 mg. of thallium per cc. of medium gives an increase in the yield of yeast of 80 per cent. Greater concentrations are toxic and lesser concentrations give less growth. Concentrations of about 10 mg. per cc. inhibit the growth of the yeast almost completely. Thallium should not be added to the medium when the yeast is to be used as a food for man because of

the extreme toxicity of this element to mammals. Inspection of the literature indicates that thallium may be one of the growth stimulants for yeast that have been referred to as a bios. It is believed that attempts to isolate and identify complex organic materials alleged to stimulate the growth of yeast will be premature and unsuccessful until the inorganic food requirements of yeast are known to be supplied completely by culture media. The importance of inorganic foods for the growth of yeast is stressed and it is suggested that part of the conflicting observations found in the literature on the growth of yeast are due to inadequate culture media and inconstant conditions of growth.

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## THE DETERMINATION OF MAGNESIUM IN BLOOD WITH 8-HYDROXYQUINOLINE

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### INTRODUCTION

The use of 8-hydroxyquinoline for the quantitative determination of magnesium in blood offers a number of advantages over the methods in current use for the estimation of this element, based on the precipitation of magnesium ammonium phosphate. According to Berg (1), who introduced the use of 8-hydroxyquinoline for magnesium analysis, magnesium hydroxyquinolate is a crystalline precipitate—greenish yellow in color—of very exact analytical composition and of a high molecular weight which makes it very suitable for gravimetric analysis. Since the compound is a phenol derivative, the methods for determining phenols, namely bromination and development of a color by means of the phenol reagent of Folin, can also be employed for analysis. Furthermore, the hydroxyquinolates of the other alkali earth elements are more soluble than the magnesium compound, so that in blood the calcium need not be removed quantitatively before analyzing for the magnesium.

The first to adopt this reagent for the estimation of magnesium in blood was Yoshimatsu (2) who developed a colorimetric method by allowing the Folin and Denis reagent to react with the hydroxyquinoline and then comparing against a standard solution of known magnesium hydroxyquinoline content, similarly treated. In checking up the Yoshimatsu method, Eichholtz and Berg (3) found it unreliable since in the presence of air when there are present traces of such heavy metals as copper and zinc, an oxidation takes place which diminishes the amount of color obtained with Folin's phenol reagent. Eichholtz and Berg modified Yoshi-

matsu's method so as to avoid traces of copper and zinc by using especially distilled water and carrying out the analyses in quartz vessels. However, the precautions and modifications developed by Eichholtz and Berg unfortunately make the colorimetric method difficult and unsuitable for general use.

Since the determination of magnesium with hydroxyquinoline by bromination does not suffer from the defects of the colorimetric procedure, and requires no extra special precautions, it seemed feasible to us to develop a method of analysis for magnesium in blood, making use of the bromination reaction. In this we have been successful, and in the following there will be described procedures for the determination of magnesium in oxalated whole blood, oxalated plasma, and in serum. While the bromination method is quite sensitive—1 molecule of magnesium being equivalent to 8 molecules of bromine—it is not as sensitive as the colorimetric method and accordingly larger blood samples are required to give the same accuracy.

When our experimental work was about completed, there appeared a paper by Bomskov (4), for the determination of magnesium in serum by hydroxyquinoline, which also employs the bromination reaction. In other respects, our method differs largely from and, we feel, is superior in point of simplicity to both those of Bomskov and of Eichholtz and Berg. These points of difference are: A single precipitation only is required to precipitate the magnesium hydroxyquinolate in a pure state; and the precipitate is isolated and washed by filtration instead of by centrifuging. In beginning our work, we were under the impression from the statements of Berg and of Eichholtz and Berg that in an ammonia-containing solution at a controlled pH and at temperatures near to boiling, magnesium could be quantitatively separated from the calcium in serum. However, we soon found that a small amount of calcium is carried down with the magnesium if it is not first at least partly removed. In this we are borne out by Bomskov, who employs two precipitations to get rid of the calcium carried along. To accomplish the same end, we resorted to the use of oxalated blood.

The authors mentioned above isolate and wash the magnesium hydroxyquinolate precipitate by centrifuging. Magnesium hy-

droxyquinolate, unlike calcium oxalate, is not well adapted for this and great care is required to avoid loss. Filtration, with use of the Kirk-Schmidt (5) micro filter, greatly simplifies this step in the analysis.

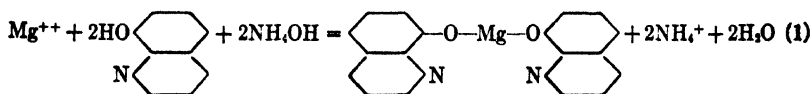
In another important point of the method we also differ from Bomskov. Bomskov adds just sufficient bromate solution from a micro burette to give a slight excess of bromine for the bromination of the hydroxyquinoline. This we feel is not altogether trustworthy. In our experiments on this point it was found that a considerable excess of bromine is needed to give a rapid and smooth bromination. Accordingly, in our procedure, the amount of bromine set free is considerably in excess of the amount necessary to brominate the hydroxyquinoline present.

In working out the analytical procedures given below, checks were made upon the methods by testing the recovery of known amounts of magnesium added to the blood samples.

#### *Analytical Procedure*

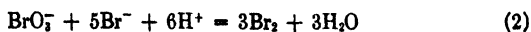
The magnesium determination is carried out on a protein-free blood filtrate, prepared either by a modification of the Folin-Wu or the trichloroacetic acid method. The filtrates are prepared in a dilution of 1:5 so that 5 ml. of filtrate represent 1 ml. of blood.

An outline of the reactions that are involved in the analytical method is given in the following equations.

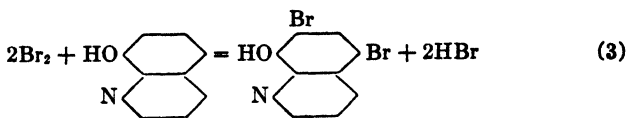


Equation 1 shows the steps of the formation of the magnesium hydroxyquinolate in an ammoniacal solution.

The bromine for the bromination is produced by permitting bromate to react with an excess of bromide in acid solution according to Equation 2.



With the bromine formed in this way, the hydroxyquinoline reacts as is indicated by Equation 3.



More bromate is added than is actually required to brominate the hydroxyquinoline and the excess is determined iodometrically by adding potassium iodide to the solution to react with the excess bromate and the liberated iodine is titrated with sodium thiosulfate. Since 4 atoms of bromine are removed in the bromination of 1 molecule of hydroxyquinoline, it is seen that 8 gm. atoms of bromine are equivalent to 1 mol of magnesium. From this it follows that 1 ml. of 0.01 M  $\text{KBrO}_3$  solution is equivalent to 0.1824 mg. of magnesium.

### *Reagents*

The following is the list of reagents and materials required for the determination.

For the preparation of the blood filtrates there are required a 10 per cent sodium tungstate ( $\text{Na}_2\text{WO}_4$ ) solution and one of 0.67 N  $\text{H}_2\text{SO}_4$  for the modified Folin-Wu and 10 per cent trichloroacetic acid for the trichloroacetic acid filtrate. In the analysis of serum there is also needed a 4.5 per cent neutral potassium oxalate solution to precipitate calcium.

To precipitate and wash the magnesium hydroxyquinolate there are required a 1 per cent 8-hydroxyquinoline solution in 95 per cent ethyl alcohol (which should be made up fresh at least every 2 weeks), 1 per cent  $\text{NH}_4\text{Cl}$ , concentrated  $\text{NH}_4\text{OH}$ , a 2 per cent  $\text{NH}_4\text{OH}$  wash solution (2 ml. of concentrated  $\text{NH}_4\text{OH}$  in 98 ml. of water), and 95 per cent ethyl alcohol.

To carry out the titration requires the following solutions: concentrated  $\text{HCl}$ , 50 per cent  $\text{KBr}$ , an exactly standardized 0.01 M  $\text{KBrO}_3$ , 20 per cent  $\text{KI}$ , 1 per cent soluble starch, and approximately 0.1 N sodium thiosulfate solution. The 0.01 M  $\text{KBrO}_3$  is prepared by dissolving 1.670 gm. of the pure dry crystalline salt in 1 liter of water at room temperature.

For the filtration, a battery of Kirk-Schmidt micro filters (5) fitted into 250 cc. Pyrex suction filtration flasks is employed. Filters with stems 2 inches long were found most suitable.

*Procedure for Oxalated Whole Blood or Plasma*

When the blood is drawn, enough neutral sodium or potassium oxalate is added to have the blood contain about 0.4 per cent oxalate. This will sufficiently precipitate the calcium so that it will not interfere further in the analysis. By the Folin-Wu method, a protein-free filtrate is prepared from the oxalated whole blood or plasma by adding to 1 volume of blood 2 volumes of distilled water, 1 volume of the 10 per cent  $\text{Na}_2\text{WO}_4$ , and then very slowly and with shaking, 1 volume of 0.67 N  $\text{H}_2\text{SO}_4$ . For whole blood, the sample is shaken up and allowed to stand after the addition of the distilled water until the change in color and decreased turbidity show that the corpuscles have been hemolyzed. A drop of concentrated ammonia added to samples that hemolyze with difficulty is helpful. The coagulum is allowed to stand for 15 minutes or more and is then either filtered or centrifuged off to obtain the filtrate. In our experience, this method gives a completely protein-free filtrate. It is important that the filtrate be protein-free since if any is left it interferes with the later precipitation of the magnesium. A trichloroacetic acid filtrate is prepared by adding to 1 volume of blood, 2 volumes of distilled water and then after taking the same precautions to obtain laking for whole blood as are described for the Folin-Wu filtrate, 2 volumes of 10 per cent trichloroacetic acid are added to coagulate the protein. When a single determination is to be made on a sample, it is our practice to prepare the filtrate by pipetting 3 ml. of the blood or plasma into a test-tube fitting into a 15 ml. centrifuge holder, then adding 6 ml. of water followed by the 3 ml. each of  $\text{Na}_2\text{WO}_4$  and  $\text{H}_2\text{SO}_4$ , or alternatively, by 6 ml. of the trichloroacetic acid. After the period of standing, the tube is centrifuged to throw down the protein and from the supernatant liquid a 10 ml. portion is pipetted off for analysis.

The 10 ml. aliquot—equivalent to 2 ml. of the original blood or plasma—of the protein-free filtrate is introduced into a 15 ml. test-tube, 1 ml. of 1 per cent  $\text{NH}_4\text{Cl}$  is added, and then 6 drops of concentrated  $\text{NH}_4\text{OH}$  followed by 1 ml. of the 1 per cent alcoholic hydroxyquinoline solution. With filtrates prepared by the trichloroacetic acid method, the concentrated ammonia added is insufficient for neutralization and this can be detected by the color of the tube having a green tint. More concentrated am-



monia is now added drop by drop until the hydroxyquinoline color in the tube changes to yellow. The contents are then mixed and the test-tube is placed in a water bath kept heated to between 70-80° for from 20 to 30 minutes. At the end of this time the filtration is carried out while the solution is still hot through the Kirk and Schmidt micro filter, gentle suction being used. The precipitate is washed first with 8 ml. of hot 2 per cent  $\text{NH}_4\text{OH}$ , then with 8 ml. of 95 per cent  $\text{C}_2\text{H}_5\text{OH}$ , and finally with another 8 ml. portion of hot 2 per cent  $\text{NH}_4\text{OH}$ . The filter is sucked dry and is then transferred to a clean filter flask. Now 8 ml. of hot concentrated  $\text{HCl}$  are added to dissolve the magnesium precipitate and allowed slowly to filter through. The filter cup is then washed out with water.

The titration is carried out in the filter flask. To the contents of the flask 1 ml. of the 50 per cent  $\text{KBr}$  is added and then slowly, with a carefully calibrated pipette, 5 ml. of the 0.01  $\text{M}$  standard  $\text{KBrO}_3$  solution. Shake gently for 1 minute to allow for the bromination of the hydroxyquinoline. Now add 1 cc. of the 20 per cent  $\text{KI}$  solution. Shake again and wash down the sides of the flask with water to give a volume of about 50 ml. To titrate the excess iodine with thiosulfate, 0.02  $\text{N}$   $\text{Na}_2\text{S}_2\text{O}_3$  is prepared by pipetting out 100 ml. of the 0.1  $\text{N}$  stock solution into a 500 ml. volumetric flask and adding water to the calibration mark. The volumetric flask is then agitated thoroughly to mix the thiosulfate solution. With the volume of blood used for the sample it will require over 10 ml. and less than 15 ml. of the diluted thiosulfate to titrate the liberated iodine. Accordingly, 10 ml. of thiosulfate are added first with a calibrated pipette and the rest of the titration is continued with a micro burette calibrated in 0.02 ml. The titration is first carried to a pale straw-color, then 20 drops of 1 per cent starch solution are added and the titration continued to the disappearance of the blue color.

The thiosulfate solution is not carefully standardized, and the accuracy of the whole method depends upon the standardization of the bromate solution. The strength of the thiosulfate is determined in each series of analyses by running a blank titration which also gives a check on the reagents used for the titration. The blank titration is carried out under the same conditions as the actual analysis in the following way.

In an Erlenmeyer flask of 200 to 300 ml. capacity, 8 ml. of concentrated HCl are added, followed by 8 ml. of water and 1 ml. of 50 per cent KBr. These are mixed and the same volume of standard bromate pipetted in as for the magnesium determinations. The flask is now shaken gently for 1 minute, 1 ml. of the 20 per cent KI solution is added, and the sides washed down as before to a volume of about 50 ml. The titration with thiosulfate is made by adding 10 ml. with a pipette and finishing up from the micro burette. The difference between the titration of the blank and the magnesium samples gives the amount used up in the bromination.

### *Serum*

The procedure for serum depends upon whether or not calcium needs to be determined. When it is not required first to determine calcium, a filtrate is prepared by pipetting into a tube fitting a 15 ml. centrifuge holder 3 ml. of serum, then 5 ml. of water. To this there are added 3 ml. of 10 per cent  $\text{Na}_2\text{WO}_4$  followed by 3 ml. of 0.67 N  $\text{H}_2\text{SO}_4$  which is added slowly; meanwhile the tube is shaken from time to time. The tube is then allowed to stand for 5 minutes. After this period, 1 ml. of 4.5 per cent potassium oxalate is added and the tube and contents are allowed to stand 2 hours to precipitate the calcium. After this period the tube is centrifuged and a 10 ml. aliquot of the filtrate is pipetted out for analysis, which, since the total volume in this procedure has been made up to 15 ml., represents a 2 ml. aliquot of serum. The rest of the analysis is the same as for oxalated plasma.

When calcium is to be determined on the serum, a trichloroacetic acid filtrate is used after the manner of Van Slyke and Sendroy (6). The filtrate is prepared as described for whole blood and plasma by adding 2 volumes of distilled water and 2 volumes of trichloroacetic acid to 1 volume of serum. 10 ml. of the filtrate, representing 2 ml. of serum, are pipetted into a test-tube for the analysis. 1 ml. of ammonia solution (containing sufficient ammonia to neutralize the trichloroacetic acid<sup>1</sup>) is added, followed by 1 ml. of saturated ammonium oxalate. After

<sup>1</sup> The ammonia solution is prepared by titrating 2 ml. of the 10 per cent trichloroacetic acid against 1:1 ammonia and then diluting the ammonia so that 1 ml. will contain the amount required for the titration.

allowing the precipitated calcium oxalate to stand for 2 hours or more, the calcium oxalate precipitate is filtered off through a Kirk-Schmidt filter, and the filtrate is caught in a test-tube placed in the filtration flask into which the tip of the filter extends. The rest of the calcium analysis is carried out as described by Kirk and Schmidt (5); namely, the precipitate is washed with 2 per cent  $\text{NH}_4\text{OH}$ , then is dissolved with 2 N  $\text{H}_2\text{SO}_4$  in a clean test-tube, and titrated with 0.005 N  $\text{HMnO}_4$  from a micro burette.

The filtrate from the calcium analysis is used for the determination of the magnesium. A 10 ml. portion of this filtrate, which represents  $1\frac{2}{3}$  ml. of serum, is pipetted into a test-tube. For the analysis, ammonium chloride, concentrated ammonia, and hydroxyquinoline are added and the rest of the determination is carried out in the same manner as is described for the trichloroacetic acid filtrate from oxalated plasma.

*Calculation*—We have already noted that in the bromination method 1 ml. of 0.01 M bromate is equivalent to 0.1824 mg. of magnesium. When an aliquot representing 2 ml. of whole blood, plasma, or serum is used, the magnesium in mg. per 100 ml. is given by the formula

$$\left( 5 - 5 \times \frac{\text{ml. Na}_2\text{S}_2\text{O}_3 \text{ (determination)}}{\text{ml. Na}_2\text{S}_2\text{O}_3 \text{ (blank)}} \right) \times \frac{0.1824}{2} \times 100$$

where 5 ml. of 0.01 M  $\text{KBrO}_3$  are used for bromination, or combining

$$\left( 5 - 5 \times \frac{\text{ml. Na}_2\text{S}_2\text{O}_3 \text{ (determination)}}{\text{ml. Na}_2\text{S}_2\text{O}_3 \text{ (blank)}} \right) \times 9.12 = \text{mg. magnesium per 100 ml.}$$

With serum filtrates on which calcium is determined, the sample for magnesium represents  $1\frac{2}{3}$  ml. of serum, so the factor in the equation will be 10.95 instead of 9.12.

#### DISCUSSION

The results of typical experiments carried out to develop and test analytical procedures are given in Table I. In Experiments 1 and 11 it is shown that the little calcium left in oxalated blood no longer interferes with the analysis, while from Experiment 3 it is seen that the amount of calcium contained in blood serum leads to too high results unless at least partially removed.

TABLE I

*Some Typical Experiments Used to Test the Accuracy of the Analytical Method for Magnesium and Calcium*

Experiment No.	Sample	No. of determinations	Magnesium <i>mg. per 100 ml.</i>	Remarks
1	Beef A			
	Plasma	3	$3.83 \pm 0.05$	Showing that calcium in oxalated blood is not enough to interfere
	" + added oxalate	4	$3.83 \pm 0.02$	
2	Plasma + 4.27 mg. Mg per 100 ml. added	4	$8.09 \pm 0.05$	Test of recovery; expected, 8.05 mg.
3	Serum, no oxalate added	4	$4.00 \pm 0.02$	Showing that calcium interferes with analysis unless partly removed
	Serum + added oxalate	4	$3.83 \pm 0.01$	
4	Fig B, whole blood Folin-Wu filtrate Trichloroacetic acid filtrate	3	$9.10 \pm 0.04$	Showing that Folin-Wu and trichloroacetic acid filtrates are equally suitable for whole blood
		6	$9.06 \pm 0.02$	
5	Whole blood + 1.2 mg. Mg per 100 ml. added (trichloroacetic filtrate)	2	$10.22 \pm 0.04$	Test of recovery; expected, 10.26 mg.
6	Plasma, Folin-Wu filtrate	2	$3.97 \pm 0.05$	Showing that Folin-Wu and trichloroacetic acid filtrates are equally suitable for plasma
	Trichloroacetic acid filtrate	2	$4.00 \pm 0.01$	
7	Serum (oxalated), Folin-Wu filtrate	3	$4.05 \pm 0.03$	Showing that Folin-Wu and trichloroacetic acid filtrates are equally suitable for serum
	Trichloroacetic acid filtrate from calcium determination	3	$4.02 \pm 0.02$	

TABLE I—*Concluded*

Experiment No.	Sample	No. of determinations	Magnesium <i>mg. per 100 ml.</i>	Remarks
8	Pig B, corpuscles* Folin-Wu filtrate Trichloroacetic acid filtrate			Showing agreement between analysis on Folin-Wu and trichloroacetic acid filtrates
		2	6.65±0.05	
		2	6.64±0.02	
9	Human serum (oxalated)	1	2.20	Test of recovery; expected, 3.82 mg.
	Human serum + 1.62 mg. Mg per 100 ml. added	2	3.85	
10	Dog W, serum (oxalated)	1	2.43	Test of recovery; expected, 4.05 mg.
	Dog W, serum + 1.62 mg. Mg per 100 ml. added	1	4.01	
11	Dog B, plasma	2	2.83	Showing that amount of calcium in oxalated blood is not enough to interfere
	" " " + added oxalate	2	2.82	

## Calcium determinations on trichloroacetic acid filtrates

			Calcium	
12	Pig B, serum Trichloroacetic acid filtrate of serum	3	11.00±0.10	Showing agreement between calcium determinations on serum and procedure on trichloroacetic acid filtrates of serum
		3	11.05±0.05	
13	Dog Gr, serum Trichloroacetic acid filtrate of serum	1	10.65	
		2	10.70	
14	Dog Gr (after parathormone injection), serum Trichloroacetic acid filtrate	2	16.10	
		2	16.20	

\* The corpuscles from 200 ml. of whole blood were washed three times with isotonic sodium chloride, and then laked and diluted to the original 200 ml. volume. From the hematocrit value of the pig blood (44.6 per cent), the corpuscle analysis, and the plasma analysis, it is readily calculated that the whole blood magnesium should be  $6.65 + 2.25 = 9.00$  mg. per 100 ml. of blood. This figure checks favorably with the value found by actual analysis.

In Experiments 2, 5, 9, and 10 it is shown that a complete recovery is obtained of the magnesium added to whole blood, plasma, or serum. From Experiments 4, 6, 7, and 8 it is seen that the modified Folin-Wu and the trichloroacetic acid filtrates yield the same analytical figures, showing both are equally suitable for the magnesium determination by this method. Finally, in Experiments 12 to 14 are given sample analyses showing that the procedure described for the determination of calcium on the trichloroacetic acid serum filtrates gives results that coincide thoroughly with the values obtained for the analysis of calcium on serum directly.

The experiments listed in Table I attest to the general accuracy and reliability of the method described here for determination of magnesium on the different blood fractions. Several hundred analyses on human and animal blood samples, which have already been carried out by us, confirm the opinion derived from the experiments in Table I on the general satisfactoriness of the method.

The results of the experiments listed in Table I, and our later analyses, already lead to the conclusion that the magnesium content of the serum and the plasma of the same blood are identical. Further, they confirm the finding of Kramer and Tisdall (7) that the red corpuscles contain more magnesium than does the blood plasma.

#### SUMMARY

A method is described for the determination of magnesium in oxalated whole blood, oxalated plasma, and in serum.

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# THE NATURE OF THE SUGAR OF NORMAL URINE

## I. THE PHENYLOSAZONES

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Despite a general interest in the osazones of normal urine, surprisingly little chemical work has been done upon them, and no careful, critical estimate of such work is available.<sup>1</sup> Hirschl (23) first noticed among the débris of the osazone test occasional irregular, thorn-apple-shaped crystals, soluble in alcohol and insoluble in water. Many others (17, 25, 28, 30, 36, 44, 45, 47, 49, 51, 53) have found crystals in the precipitate from every normal urine examined, and the frequency of the thorn-apple variety has been established (1, 6, 13, 25, 28, 30, 32).

As early as 1905 McEwen (32) made a clear distinction between the two kinds of crystals previously noted by Frank; namely, phenylglucosazone and the thorn-apple crystals, which McEwen described as "much smaller crystals, in the form of boat-shaped staves with centrally disposed radiating spiculæ, like the seed-pod of *Datura stramonium*, and likened, save for their color, to crystals of ammonium urate." Geelmuyden (15) gave the name "physiological sugar" to the non-glucose material from which the thorn-apple crystals originated. In his Fig. *f* he pictured rosettes that were apparently phenylglucosazone, but after mechanical fracture were found to consist of thorn-apple (sword-like) crystals. This circumstance should have served as a warning to microscopists who have been identifying such rosettes as phenylglucosazone, yet the finding has been generally ignored. While Geelmuyden said that the thorn-apple crystals were more reddish than phenylglucosazone, Höst (24) described the latter as the more deeply colored. Höst's results and descriptions lead

<sup>1</sup> For general reviews consult Neubauer-Huppert (37) and Neuberg (38).



us to believe that he was confused by the false phenylglucosazone of Geelmuyden.

Malmros' (30) photographs of urine osazones may be roughly divided into the two types already mentioned. His Plate IV is a beautiful illustration of the effect of graded additions of glucose upon the crystal form, first shown by Frank (13). Here one may note the poorly formed crystals of the original urine, the intermediate thorn-apple types, and the ultimate sprays and sheaves of phenylglucosazone. At times Malmros recrystallized his crude osazone precipitate from alcohol-water mixtures, getting broad needles, where none was visible originally.

Hassan (22) also fractionated the crude osazone precipitate by recrystallization. The preponderating physiological non-glucose component consisted of broad, flat, orange-yellow needles with gradually tapering ends, or blunt, spear-shaped points, tending to form irregular rosettes that resembled phenylmaltosazone. These crystals were soluble in hot water, 20 per cent alcohol, and alcohol-ether mixtures.

While a substance with the melting point of phenylglucosazone has occasionally been prepared from something in normal urine (2, 5, 19, 21, 22, 34, 36, 46, 51, 55, 56), the usual result has been the production of rather impure crystals which have been variously designated as phenylosazones of glycuronic acid (5, 11, 16, 17, 21, 23, 49), isomaltose (3, 29, 46), maltose (30), pentose (14, 18, 25), or physiological sugar (15, 22, 24, 50). The osazones from urine previously hydrolyzed by Cammidge's procedure are excellent examples of troublesome mixtures. Contrary to the findings of Cammidge (9) and Pekelharing and van Hoogenhuyze (47), Grimbert and Bernier (21), Smolenski (58), and Neuberg (39) made the osazone from normal urine and unhydrolyzed urine and separated it into a high melting fraction and the water-soluble fraction of Cammidge. (See also Willcox (59).)

Realizing that a formation of mixed crystals (8, 20, 41) would account for the great confusion, we examined all original descriptions carefully and were surprised to find how many of these urinary osazones were obviously mixtures, with vague melting points and properties that attested the presence of gross impurities. The original communications of Baisch (2, 3) and of Pavy and Siau (46), which have often been cited as proof of the presence

of isomaltose in normal urine, display these facts. We believe with Salkowski (54) and Hassan (22) that most urinary phenylosazones were mixtures, whose physical properties were of little significance.

It seems curious to us that so little attention has been paid to the very suggestive, now almost forgotten work of Jaffé (26) and Milrath (33). They found that 1-phenylsemicarbazide separates as round, yellow, wart-like masses of needles from urine which has been heated with phenylhydrazine and acetic acid. Schulz (57) disposed of their findings by saying that the fear of confusion of glucose with urea is an exaggerated one, since human urine contains too little urea. This estimate of the possible danger from phenylsemicarbazide may have misled many. At least, the only further experimentation was that of Porcher (48), who noted the retarding effect of urea and of Liebig's extract upon phenylglucosazone formation. In his experiments, whenever urea was added to glucose solution, the resulting osazone (actually much phenylsemicarbazide, or mixed crystals) redissolved upon heating the solution. Porcher did not recognize this as phenylsemicarbazide formation.

#### EXPERIMENTAL

In the course of our investigation of the sugar of normal urine we prepared several gm. of crystalline osazone from the night urines of dozens of normal persons. The method of preparation was essentially that of Hassan (22). 1 to 3 gm. of a mixture of 1 part of phenylhydrazine hydrochloride and 2 parts of sodium acetate were used for every 20 cc. of prepared urine, previously adjusted to pH 6.7. The mixture was heated in the boiling water bath for 1 hour, and allowed to cool in the bath for 12 to 15 hours. It was then centrifuged and the precipitate washed three times with ice water and then dissolved in 25 per cent ethyl alcohol solution. The solution was extracted three times with equal volumes of ether, and the latter was evaporated on the water bath. After adding an equal volume of water to the residual fluid, crystallization was allowed to take place in the refrigerator. The accumulated crystals were recrystallized from 3 per cent aqueous pyridine solution, the phenylhydrazine derivative separating as rosettes.

The preparations used in the above procedure were either bone-black filtrates of urine, as recommended by Hassan, or partly purified extracts of urine made by evaporating the urine at 60° under reduced pressure, extracting the residue with absolute methyl alcohol, evaporating the extract under reduced pressure, dissolving this residue in distilled water, adding barium hydroxide solution to pH 7, and removing barium from the filtrate by means of sulfuric acid solution. By using only night samples of urine we expected to increase the yield of osazone from the uroketose (10) and to reduce the danger of alimentary mellituria to a minimum.

At first our products seemed to be phenylpentosazones, but we hesitated to regard them as such because of our inability to prepare the corresponding osazones from pure *l*-arabinose and *d*-xylose by the same process. Later we realized that continued recrystallization of the urinary osazones had raised their melting points to the vicinity of the corresponding semicarbazides. Thus, the first products from aqueous pyridine melted between 148–165°. After three or four recrystallizations, a dozen different preparations melted between 160–168°. Our purest material, recrystallized six times, was optically inactive and melted at 170–172°. These temperatures were determined in the Roth apparatus. They were carefully checked against known materials and have been corrected. Mixing our material with Eastman's pure phenylsemicarbazide resulted in no change of melting point, while mixing it with pure phenylxylosazone caused the melting point to be greatly lowered.

The appearance of our derivative was microscopically identical with that of pure phenylsemicarbazide. Most authorities describe the latter as plates or leaflets, but Holmgren's description of his thorn-apple crystals from urine gives a much better picture of their actual appearance. He says that they were strange yellow crystals, arranged in regular balls which seemed to consist of needles, and yet when seen from the side, they were plate-like and had the form of lancet-like scales. After several recrystallizations they became still more needle-like. When dry chloroform is used as a solvent, both pure phenylsemicarbazide and our urine derivative crystallize as perfectly white rectangular plates, with no rosettes. By Jaffé's procedure we converted our substance into the corresponding nitroso derivative. This proved to be

identical with that prepared from pure phenylsemicarbazide in every respect.

Micro analysis of our phenylhydrazine derivative by the Research Service Laboratories, New York, gave the following results.

4.985 mg. of substance, dried to constant weight *in vacuo*, gave 10.210 mg. of CO<sub>2</sub> and 2.665 mg. of H<sub>2</sub>O; 4.899 mg. gave 1.191 cc. of N<sub>2</sub> at 26° and 758 mm.

	C per cent	H per cent	N per cent
Found.....	55.85	5 98	27.67
Theory for phenylsemicarbazide.....	55 60	6 00	27 81
“ “ phenylpentosazone.....	61.97	6 43	17.02

Urinary *p*-nitrophenylhydrazine and *p*-bromophenylhydrazine derivatives were also obtained, the former as orange-yellow needles, melting at 194–202°, and the latter as colorless, acicular needles, melting with decomposition at 226–228°. Pure *p*-nitrophenylsemicarbazide melts at 211–212° and *p*-bromophenylsemicarbazide at 226° with decomposition. The further recrystallization of our *p*-nitrophenylsemicarbazide was not attempted because of the small amount prepared. We also experimented with  $\alpha$ - and  $\beta$ -naphthylhydrazines, *m*-nitrophenylhydrazine, methylphenylhydrazine, diphenylhydrazine, and 2,4-dinitrophenylhydrazine, but these gave only oils or amorphous products not easily crystallized. Recognizable hydrazones could not be obtained by means of the hydrazines mentioned in this paper, either by classical methods, or by extraction of the mixture with ether, etc. Not only was this true for urine, but also for the concentrated, purified syrups which we have made from normal urine by a process soon to be published.

Jaffé (26) found that 2 per cent solutions of urea gave visible phenylsemicarbazide crystals, the yield being about 85 per cent of the theoretical in the course of 24 to 48 hours. He suspected the presence of phenylsemicarbazide in urinary osazones and was able to prepare an appreciable amount from normal dog urine. He was unable to form a definite conclusion about human urine.

We have secured crystals of discolored phenylsemicarbazide by the customary osazone procedure from artificial urines containing 2.3 per cent urea. The artificial urine was prepared by mixing 1 cc. of each of the following with 2 cc. of 2.3 per cent potassium

acid phosphate and 4 cc. of 0.4 per cent disodium urate·H<sub>2</sub>O. The mixture was adjusted to pH 6.5.

<i>per cent</i>		<i>per cent</i>	
2.0	alanine	21.0	magnesium chloride·6H <sub>2</sub> O
1.5	ammonium chloride	2.7	potassium chloride
4.4	“ sulfate	1.3	“ nitrate
8.0	calcium chloride·H <sub>2</sub> O	0.16	“ thiocyanate
0.15	“ lactate·5H <sub>2</sub> O	19.0	sodium chloride
0.3	<i>p</i> -cresol	1.3	“ hippurate
2.0	creatinine	46.0	urea

When the urea concentration was doubled the yield of crystals was greatly increased. When urea was omitted from the mixture these crystals were not found. The yield from the dilute mixture was uncertain and variable, but comparatively large amounts of phenylsemicarbazide, identified by its crystal form and melting point, could always be separated from the supernatant liquid by means of alcohol-ether extraction. This was also true for normal urine. The original crystals of the osazone precipitate are often very misleading, but they assume their characteristic shape after being recrystallized.

It is therefore obvious that 1-phenylsemicarbazide will be produced by the phenylhydrazine test from every normal urine. It is merely its massive crystallization, observed by Jaffé and Milrath, which requires longer heating. We have been able to show that urea and sodium hippurate, especially the former, increase the solubility of phenylsemicarbazide in water. Sodium chloride and ammonium sulfate, in larger concentrations, have an appreciable salting out effect, thus aiding the crystallization of phenylsemicarbazide, which has a tendency to form supersaturated solutions. It can easily be seen that the actual degree of crystallization of phenylsemicarbazide in the usual osazone test will be the result of the interplay of many factors. We wish to enumerate the following.

*Concentration of Urine*—As urine becomes concentrated phenylsemicarbazide formation is hastened by the greater urea concentration. The increased concentration of salts will aid its crystallization. Several investigators (4, 13, 25, 42, 49) have noticed that the sensitivity of the osazone test is increased by concentrating the urine.

*Acidity of Test Mixture*—Certain modifications of von Jaksch's test (27), such as Neumann's (40), are said to be more sensitive (31), but they also employ a liberal amount of acid which, hastening the production of cyanic acid from urea, produces larger yields of phenylsemicarbazide. Holmgren (25) showed that urines which had been made slightly alkaline gave no crystals, but he attributed this to the destruction of sugar by alkali.

*Extent of Heating and Subsequent Cooling*—Moritz (35) noticed that prolonged heating increased the sensitivity of the test. Since the heating period has been gradually lengthened from 15 minutes (27) to 2 hours (22) the errors from phenylsemicarbazide have been correspondingly increased.

*Previous Formation of Cyanic Acid by Hydrolytic Procedures*—We have been able to show very clearly that previous hydrolysis of artificial urine, or of dilute urea solutions, by Cammidge's procedure, results in a tremendously increased yield of phenylsemicarbazide in the usual osazone test. We have assumed that the production of cyanate is the cause, since potassium cyanate solutions act just like Cammidge's hydrolysate. The phenylsemicarbazide separating from such test mixtures is well crystallized in the form of rosettes. It is quite obvious from these experiments that 1-phenylsemicarbazide is one of the chief components of Cammidge's osazone.

Cammidge stated that one peculiarity of his osazone was its marked solubility in 33 per cent sulfuric acid solution. Phenylsemicarbazide is much more soluble in the latter than phenylglucosazone is. It is also quite soluble in chloroform, which accounts for Moritz' observation (35). Its marked solubility in hot water may well account for the more soluble constituent of many osazone mixtures (12, 21, 22, 25, 30, 49).

In artificial urine mixtures and in phenylsemicarbazide solutions we have observed practically every form of osazone crystal described by others. By conducting the osazone test upon solutions containing both glucose and phenylsemicarbazide, we have found forms like the mixed crystals of Hassan (22), and have noted the growth of one of these substances fixed solidly upon a matrix of the other, as noted by several with normal urine (15, 22, 24, 30). We conclude that the physical form, composition, and melting point of osazones from normal urine, with its great urea

content, are not trustworthy. While phenylglucosazone appears to form mixed crystals with phenylsemicarbazide under certain circumstances, it is nevertheless quite insoluble in the latter. Acid ammonium urate crystals, while similar to those of phenylsemicarbazide in some respects, do not appear as mixed crystals and can scarcely be confused with either of the other substances.

After urine has been fermented for many hours (24, 30, 32), or after it has been allowed to undergo spontaneous ammoniacal fermentation (22, 30), the urea concentration, and hence the phenylsemicarbazide formation, will be greatly reduced. Also we would expect Patein-Dufau filtrates of urine to yield little phenylsemicarbazide, provided the urea has been properly precipitated. Therefore the osazones from such filtrates should be the purest and should have the sharpest melting points, if nothing other than phenylsemicarbazide is a disturbing factor. Actually, these osazones have had the least sharp melting points and were obviously mixtures (5, 11, 12, 17, 21, 43). There are two reasons for this circumstance. First, some investigators have not made their precipitating mixtures definitely alkaline during the urea removal. It is true that Patein and Dufau's directions (42) call merely for neutralization, or for the production of a slight acidity, but Gilbert and Baudouin (17) demonstrated many years ago that greater care must be exercised. Hence there must have been some urea present in certain of these preparations. After concentration of the filtrate some phenylsemicarbazide would be formed. In other instances it appears that the urea has been carefully precipitated and we are forced to the conclusion that there is a second prolific source of error in the osazone test. Recently we have been fortunate enough to recognize the origin of this second interfering substance and will give details concerning it at another time. Suffice it to say that osazone preparations from Patein-Dufau filtrates of urine are far less trustworthy than usually supposed.

While we have called attention to these errors in the osazone test we also concede the frequent production of a high-melting phenylhydrazine derivative, probably phenylglucosazone, from something in normal urine, but not necessarily from the reducing sugar. Our personal experience, during 5 years of experimentation, has

been that the compounds of phenylhydrazine and the uroketose, if they exist at all, are very difficult to prepare. It is true that we have obtained both crystalline and amorphous derivatives of various hydrazines from purified urine preparations, but never with anything approaching the ease with which crystals may be obtained from normal urine itself. If there is so much difficulty in preparing a crystalline derivative from the more concentrated, much purer sugar syrups, how much more difficult will it be to get such substances from the original urine? The oft quoted work of Breul (7), based as it was upon the amount of the crude osazone precipitate made by lengthy heating in the presence of generous acidity, loses much of its significance. Breul's values were merely fortuitous, since we now know that his precipitates must have contained much that was not osazone. (See also Raimann (52).) Whether the precipitate from normal urine contains any true osazone remains to be determined.

For clinical purposes the heating period in the phenylhydrazine test should not exceed an hour. Dilution of the urine with an equal volume of water, as recommended by Frank, and the use of Patein-Dufau reagent are good procedures, but this dilution results in diminished sensitivity for glucose, and the use of mercuric salts is expensive. Probably the best practical means of differentiating phenylglucosazone from phenylsemicarbazide is to dissolve the latter in hot water. For this purpose, conduct the osazone test in a centrifuge tube, examine the centrifuged residue not later than 12 hours after the heating, and confirm the presence of phenylglucosazone by adding to the residue a few cc. of water, warming, and stirring thoroughly on the water bath. Examine the centrifuged residue microscopically. If typical crystals still remain the test may be considered positive. To confirm the presence of phenylsemicarbazide in the water extract, add  $\frac{1}{2}$  volume of 95 per cent ethyl alcohol and extract the mixture with an equal volume of ether. Remove the ether solution and evaporate it to dryness. Dissolve the residue in the smallest possible amount of 2 per cent warm hydrochloric acid solution. To this add saturated sodium nitrite solution, drop by drop, until the peculiar, characteristic crystals of the nitroso derivative can be distinguished with a microscope.



## SUMMARY

One of the principal components of the osazone precipitate of normal urine has been identified as 1-phenylsemicarbazide. Its formation from urea and its subsequent crystallization have been studied in artificial urine. The effects of the concentration of urine, the acidity of the test mixture, the extent of heating, and the previous formation of cyanic acid by hydrolytic procedures have been considered. The presence of phenylsemicarbazide in osazone precipitates has necessitated a revaluation of previous microscopic and analytic findings. Cammidge's test appears to involve the action of hydrochloric acid upon urea. A method is suggested for distinguishing between phenylglucosazone and phenylsemicarbazide in the usual osazone test.

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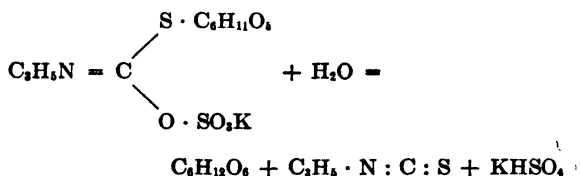
## NOTE ON MYROSIN

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Sinigrin, the thioglucoside of black mustard seed, is split by its specific enzyme into glucose, allyl isothiocyanate, and potassium bisulfate (1).



Various names have been suggested for the enzyme: myrosin (2), myrosinase (3), and sinigrinase (4). It has been fully established that myrosin is restricted to splitting thioglucosides and does not attack ethereal sulfates (5), but there is still comparatively little known about the exact nature of its action. Von Euler (6) studied the enzyme, using its power to split off sulfuric acid as a measure of its activity. He states that experiments designed to ascertain the pH range of the enzyme did not yield satisfactory results on account of the sulfuric acid which is split off during the experiment. Neuberg (5) neutralized the split off sulfuric acid by adding calcium carbonate to the mixture, but this has the disadvantage that the hydrolysis progresses very slowly, so that complete splitting was accomplished only after 12 days. We overcame these difficulties by determining the split-products by micro methods, which allowed us to use very small amounts of sinigrin. The little sulfuric acid that was split off did not change the reaction of a relatively large amount of buffer solution. The pH remained constant for 28 hours and showed only a difference of 0.2 after 52 hours. The enzyme is active over

a wide range of pH, with no clearly defined optimum of activity (Table I).

Von Euler (6) suggests that the enzyme consists of two entities, a glucosidase, the action of which is apparent within 15 minutes (identified by the smell of mustard oil), and a sulfatase which

TABLE I

*Relation of Quantity of Glucose and of Sulfur Split off from Sinigrin to pH*

pH...	Glucose								Sulfur				
	4.4	5.4	6.2	6.4	6.6	6.8	7.0	7.4	5.4	6.2	6.6	6.8	7.0
hrs.	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
1	56	62	61	64	62	61	63	64	19	20	19	19	19
4	64	65	66	65	64	66	66	65	64	64	64	64	64
28	62	63	63	62	62	62	64	62	100	98	99	100	98
52	62	60	62	62	62	61	63	61					

TABLE II

*Relation of Quantity of Glucose and of Sulfur Split off from Sinigrin to Length of Time*

pH...	Glucose			80 mg. sinigrin 8 " myrosin 10 cc. buffer solution			500 mg. sinigrin 50 " myrosin 20 cc. buffer solution	
				Sulfur				
	4.4	6.4	7.4	4.4	6.4	7.4	7.2	
hrs.	per cent	per cent	per cent	per cent	per cent	per cent	per cent	
$\frac{1}{2}$	20	21	20	4	5	4		
$\frac{1}{2}$	28	35	32	8	9	9	10	
1	56	64	64	20	20	19	15	
2	64	64	64	38	38	40	28	
4	64	65	65	67	67	66	66	
6	62	62	61	88	87	89	84	
24							99	
28	62	62	61	98	100	100		
52	62	62	61	98	100	100		

begins to act only after  $7\frac{1}{2}$  hours. We were able to show that though it seems probable that myrosin consists of two components they both start to act at the same time (Table II).

The assumption that myrosin is composed of two units is sup-

ported by the fact that the amount of myrosin required to split off the optimal quantity of glucose from sinigrin is greater than the amount necessary for the splitting off of the optimal quantity of sulfur (Table III).

The maximal yield of glucose obtainable from sinigrin does not exceed 66 per cent of the theoretical total present in the thioglucoside.

TABLE III

*Relation of Quantity of Glucose and of Sulfur Split off from Sinigrin to Ratio of Enzyme and Substrate*

Ratio, sinigrin to myrosin....		40 mg. sinigrin 10 cc. buffer solution, pH 7.2							
		Glucose							
		1:1	1:0.8	1:0.6	1:0.4	1:0.2	1:0.1	1:0.05	1:0.025
hrs.	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
1	62	59	46	35	5	4	2	2	
2	64	63	59	58	26	6	5	2	
4	65	63	59	58	40	30	8	2	
6	61	60	57	57	44	36	20	5	
28	61	60	57	57	56	52	50	38	

Ratio, sinigrin to myrosin....		500 mg. sinigrin 20 cc. buffer solution, pH 7.2						
		Sulfur						
		1:1	1:0.2	1:0.15	1:0.12	1:0.1	1:0.05	1:0.02
hrs.	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
2	70	49	27	28	28	12	12	
4	72	66	66	67	66	28	14	
6	100	84	82	83	84	28	14	
24	100	100	100	100	100	79	35	

side. The splitting off of sulfur, however, proceeds until the theoretically possible yield has been obtained.

#### EXPERIMENTAL

Sinigrin was prepared from black mustard seed, according to the method of Hérissé and Boivin (7), with slight modifications.

Boil 1 kilo of powdered black mustard seed for 20 minutes with 10 liters of 75 per cent redistilled commercial acetone. After

cooling, express, filter, and concentrate in a vacuum to about 2 liters. Water is added twice during the distillation in order to remove the last traces of acetone. Filter, add 20 gm. of fresh yeast, and allow to ferment for 2 to 3 days. Neutralize the fermented liquid by heating on the water bath with 15 to 20 gm. of  $\text{CaCO}_3$ , filter, and concentrate in a vacuum to a syrup. Reflux  $\frac{1}{2}$  hour with 3 liters of 83 to 85 per cent alcohol, allow to stand for 24 hours, filter, and completely distil off the alcohol in a vacuum. Reflux the residual extract 6 to 7 times with 250 to 300 cc. of 95 per cent alcohol; filter each fraction only after standing for 24 hours in contact with the heated extract. After inoculation with sinigrin crystals, crystallization occurs in all fractions. Recrystallize from 82 to 83 per cent alcohol (10 cc. for 1 gm. of sinigrin).

Some difficulties were encountered in producing the first crystals needed for inoculation, without which it seemed impossible to induce the extract to crystallize. They were finally obtained by very slow concentration of the first fraction in a vacuum desiccator. After inoculation crystallization readily occurs. Myrosin was prepared according to the method of Braecke (8).

Extract 500 gm. of powdered white mustard seed with 1500 cc. of water for 5 hours at  $40^\circ$ . Express through canvas, and precipitate the mucilage with the same volume of 80 per cent alcohol. Filter, and precipitate the filtrate with 2.5 volumes of 90 per cent alcohol. Filter or centrifuge, wash with alcohol and ether, dry in a vacuum, and powder.

The enzyme was stored in a vacuum desiccator, and kept its full activity for several months.

The split off glucose was determined according to the method of Folin and Malmros (9). 40 mg. of sinigrin and 40 mg. of myrosin, dissolved in 20 cc. of buffer solution (10), were used in all experiments, except in those designed to determine the optimal ratio of enzyme and substrate, where 40 mg. of sinigrin were treated with varying amounts of myrosin. The experimental mixture was kept at a temperature of  $37^\circ$ . Portions containing 0.08 mg. of sinigrin were withdrawn for each determination.

Sulfur determinations were made according to the method of Hubbard (11). Since the presence of phosphates interferes with this method, the phosphate which had been introduced into the experimental mixture by the buffer solution was removed accord-

ing to the method of Fiske (12). 80 mg. of sinigrin and 8 mg. of myrosin were dissolved in 10 cc. of buffer solution in the experiments designed to show the influence of pH and length of time on the hydrolysis of the thioglucoside. In another group of experiments 500 mg. of sinigrin and 50 mg. of myrosin were dissolved in 20 cc. of buffer solution, but the hydrolysis progressed at approximately the same rate as in the experiments with the smaller amount of substrate. In the experiments designed to determine the optimal ratio of enzyme and substrate for the splitting off of sulfur from sinigrin, 500 mg. of sinigrin were treated with varying amounts of myrosin. Suitable controls were run to exclude errors that might arise from the presence of split-products.

#### SUMMARY

Myrosin contains two units, a glucosidase and a thiosulfatase. Both units are active over a wide range of H ion concentration with no clearly defined pH optimum. The two components of the enzyme start to act on the substrate at the same time. Larger amounts of myrosin are necessary for the optimal splitting off of glucose than for the maximal splitting off of sulfur. The splitting off of glucose stops at 66 per cent of the theoretical amount present in the thioglucoside, while the splitting off of sulfur progresses up to 100 per cent.

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12. Fiske, C. H., *J. Biol. Chem.*, **47**, 59 (1921).





## STRUCTURE OF $\gamma$ -GLYCOSIDES

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Although the ring structures of the fully methylated sugars have been established beyond dispute, those of the unsubstituted sugars, on the other hand, continued to be a matter of discussion. Recently, however, the normal glycosides of certain non-substituted sugars have been demonstrated by Haworth and Hirst,<sup>1</sup> Müller,<sup>2</sup> and Isbell<sup>3</sup> to possess the pyranose structure, thus leaving only the  $\gamma$ -glycosides of these sugars open to question. For these  $\gamma$ -glycosides the alternative structures which are to be considered are the  $<1, 3>$  or propyleneoxidic and the  $<1, 4>$  or butyleneoxidic forms. It should be possible to solve the problem by a study of the glycoside formation of a 3-monomethyl and a 4-monomethyl sugar. Of these the former cannot form a propyleneoxidic ring and the latter cannot form a butyleneoxidic ring. A study of the types of glycosides formed in the two cases should therefore indicate the ring form of the  $\gamma$ -glycosides.

In a publication by Levene and Dillon<sup>4</sup> it was shown that 3-methylglucose does, in fact, form a typical  $\gamma$ -glucoside and at about the same rate as does glucose. Methylation of this  $\gamma$ -glucoside led to the ordinary 2, 3, 5, 6-tetramethylglucofuranose, showing that position (3) was not involved in the formation of the  $\gamma$ -glucoside. It was stated at that time that work on a sugar substituted in position (4) was in progress in order to complete the evidence. The results of this work are presented in this communication.

4-Methylglucoheptose was prepared from 3-methylglucose

<sup>1</sup> Haworth, W. N., and Hirst, E. L., *J. Chem. Soc.*, 2615 (1930).

<sup>2</sup> Müller, A., *Ber. chem. Ges.*, **64**, 1820 (1931).

<sup>3</sup> Isbell, H. S., *Bureau Standards J. Research*, **5**, 1179 (1930); **7**, 1115 (1931).

<sup>4</sup> Levene, P. A., and Dillon, R. T., *J. Biol. Chem.*, **92**, 769 (1931).

through the cyanhydrin synthesis, and was subjected to treatment with methyl alcoholic hydrogen chloride. In Table I are given the results obtained for the glycoside formation of this sugar. From Table I it may be seen that only a normal glycoside is formed from this sugar under conditions which lead to the production of both a normal and a  $\gamma$ -glucoside in the cases of glucose,

TABLE I  
*Sugar Distribution during Glycoside Formation at 25°*

Glucose				Tetramethylglucopyranose				Tetramethylglucofuranose			
Time	Free sugar	Glucoside		Time	Free sugar	Glucoside		Time	Free sugar	Glucoside	
		$\gamma$	Normal			$\gamma$	Normal			$\gamma$	Normal
hrs.	per cent	per cent	per cent	hrs.	per cent	per cent	per cent	hrs.	per cent	per cent	per cent
0	101	-3	2	0	101	1	-2	0	81	25	-6
1	76	20	4	2	97	1	2	0.33	15	90	-5
3	48	47	5	5	94	1	5	0.67	5	96	-1
7	26	66	8	24	71	2	27	1	4	96	0
24	12	68	20	72	35	0	65	5	4	99	-3
48	10	63	27	336	2	0	98	24	2	100	-2
2-Methylglucose				3-Methylglucose				4-Methylglucoheptose			
0	105	-7	2	0	102	0	-2	0	104	0	-4
1	90	9	1	1	80	19	1	1	98	3	-1
3	68	21	11	3	52	50	-2	3	87	-2	15
7	47	32	22	7	25	79	-4	7	66	1	33
24	14	45	44	24	8	89	3	24	16	4	80
48	11	49	43	48	7	87	6	48	4	1	95
$\alpha$ -Glucoheptose											
24	6	48	46								
48	2	24	74								

2-methylglucose, and 3-methylglucose. However, as may be seen from Table I, the rate of glycoside formation is not the same as in the case of tetramethylglucopyranose.

In order to elucidate the structure of this glycoside it was isolated and methylated. Hydrolysis of the resulting pentamethylmethylheptoside gave a pentamethylheptose of which the  $\alpha$

form was isolated. Haworth, Hirst, and Stacey<sup>5</sup> had described the  $\beta$  form of this same sugar. Oxidation to the acid and conversion to the lactone gave a product identical with that described by Haworth and shown by him to possess the  $<1, 5>$  ring. Thus there can be no doubt but that the glycoside formed from the 4-methylglucoheptose has the  $<1, 5>$  lactal ring. *The furanose structure thus may be more definitely assigned to the  $\gamma$ -glycosides of the unsubstituted sugars.*

As a further point of interest in connection with the problem, there may be mentioned the results on the glycoside formation of the non-substituted glucoheptose. Due to the insolubility of this sugar in methyl alcohol, it is difficult to secure as clear cut information as in the cases of the more soluble sugars but it is evident from the analytical data in Table I and from the optical data in Fig. 1 that the non-substituted glucoheptose forms two glycosides, a  $\gamma$  and a normal, as would be expected.

The method employed in determining the proportions of  $\gamma$  and normal glycosides consisted in subjecting the mixture of the two to mild acid hydrolysis, and determining the amount of reducing sugar present before and after such hydrolysis. From these data the proportions of normal glycoside,  $\gamma$ -glycoside, and free sugar may be calculated. The limitations of the method as well as the exact manner of applying it have been discussed in a previous publication<sup>6</sup> and need not be repeated here. It is sufficient to state that although not truly quantitative, the method gives a good first approximation—quite satisfactory for the present case. Taken with the supplementary evidence obtained from the optical data, the method is sufficiently reliable for the elucidation of the progress of the formation of the  $\gamma$  and of the normal glycosides.

## EXPERIMENTAL

### *Materials*

The glucose used was an anhydrous, reagent grade. 2-Methylglucose was prepared through the dibenzylmercapto derivative

<sup>5</sup> Haworth, W. N., Hirst, E. L., and Stacey, M., *J. Chem. Soc.*, 2864 (1931).

<sup>6</sup> Levene, P. A., Raymond, A. L., and Dillon, R. T., *J. Biol. Chem.*, 95, 699 (1932).

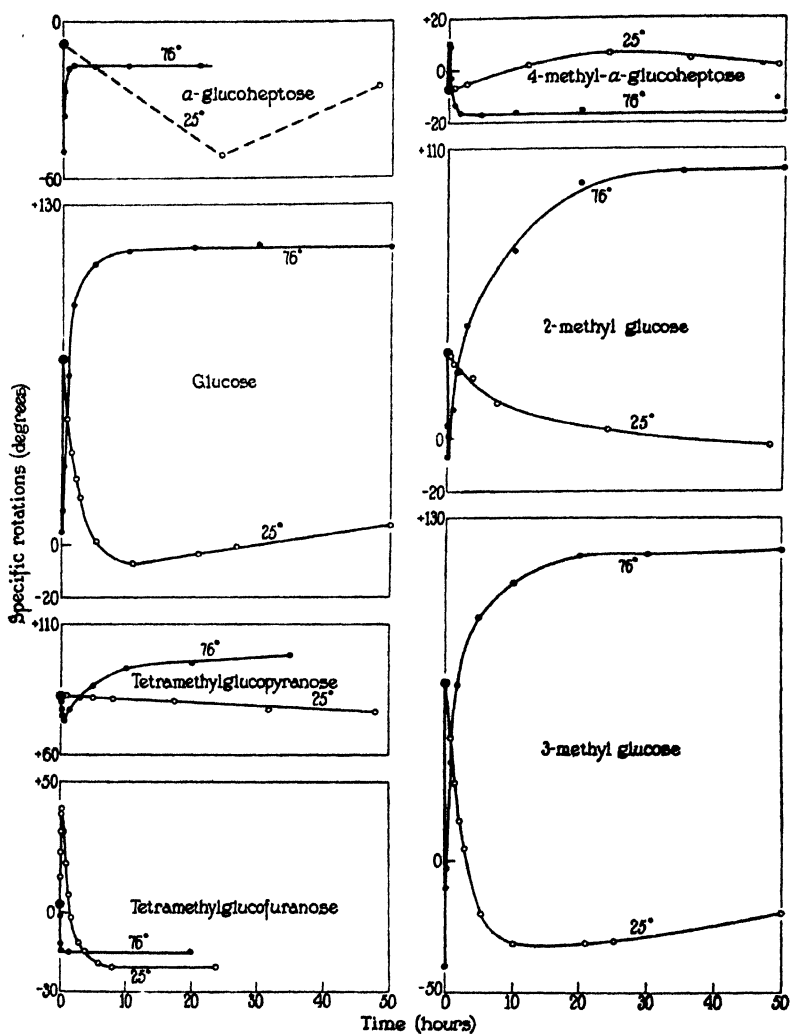


FIG. 1. Optical rotation during glycoside formation

according to the procedure of Pacsu.<sup>7</sup> 3-Methylglucose was prepared by hydrolysis of 3-methyldiacetoneglucose.<sup>8</sup> Tetramethylglucopyranose was made by methylating methylglucoside, followed by hydrolysis of the glucoside.<sup>9</sup> Glucoheptose was prepared from glucose by the cyanhydrin synthesis followed by hydrolysis and reduction with sodium amalgam.<sup>10</sup> All of these substances were purified by several recrystallizations from appropriate solvents.

For preparation of tetramethylglucofuranose,<sup>11</sup> glucose was converted to the  $\gamma$ -glucoside by standing 24 hours at room temperature in methyl alcohol containing 1 per cent of hydrogen chloride. The mixture was neutralized with sodium methylate and concentrated to a syrup. This was extracted with ethyl acetate, the solution concentrated to a syrup under reduced pressure, and this syrup was methylated with methyl iodide and silver oxide. The product was isolated and distilled three times under greatly reduced pressure. Subsequent analyses indicated this material to contain 15 per cent of glucopyranoside.

*4-Methylglucoheptose*—3-Methylglucose was treated with hydrogen cyanide and the cyanhydrin was hydrolyzed as previously described.<sup>12</sup> The recrystallized 4-methylglucoheptonic lactone was reduced with sodium amalgam in the usual manner, 10 gm. of the lactone and 150 gm. of a 2½ per cent amalgam being employed. After removing the mercury, the mixture was made alkaline with sodium hydroxide and more of this reagent was added from time to time until the mixture remained alkaline for half an hour. It was then just neutralized with sulfuric acid and concentrated under reduced pressure to a small volume. Alcohol was added to precipitate sodium sulfate and the sodium salt of the unreduced acid. The solution was concentrated again to a small volume and alcohol was again added. On concentrating this filtrate and adding alcohol the material soon crystallized. The yield of once recrystallized material was 10 gm. from 50 gm. of lactone.

<sup>7</sup> Pacsu, E., *Ber. chem. Ges.*, **58**, 1455 (1925).

<sup>8</sup> Irvine, J. C., and Scott, J. P., *J. Chem. Soc.*, **103**, 568 (1913).

<sup>9</sup> Purdie, T., and Irvine, J. C., *J. Chem. Soc.*, **83**, 1021 (1903).

<sup>10</sup> Fischer, E., *Ann. Chem.*, **270**, 72 (1892).

<sup>11</sup> Irvine, J. C., Fyfe, A. W., and Hogg, T. P., *J. Chem. Soc.*, **107**, 524 (1915).

<sup>12</sup> Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, **60**, 173 (1924).

The analysis corresponded to a methylheptose.

4.431 mg. substance: 6.931 mg.  $\text{CO}_2$  and 2.835 mg.  $\text{H}_2\text{O}$

5.473 " " : 5.765 " AgI

$\text{C}_6\text{H}_{12}\text{O}_7$ . Calculated. C 42.84, H 7.20, OCH, 13.83

Mol. wt. 224.1. Found. " 42.65, " 7.15, " 13.90

The product sintered at  $145^\circ$  and underwent a preliminary melting at  $158$ – $160^\circ$  but did not melt to a clear liquid below  $185^\circ$ . The rotation, in water, was

$$[\alpha]_D^{20} = \frac{-0.52^\circ \times 100}{2 \times 1.0} = -26.0^\circ \text{ initial}$$

and 
$$[\alpha]_D^{20} = \frac{-0.30^\circ \times 100}{2 \times 1.0} = -15.0^\circ \text{ at equilibrium}$$

An aqueous solution of 1.0 gm. of the methylheptose was mixed with a glacial acetic acid solution of 2 gm. of phenylhydrazine and heated on the steam bath. An osazone formed, but slowly. In order to complete the crystallization, the mixture was allowed to stand overnight in the refrigerator. The osazone was filtered off and dissolved in alcohol, in which it was very soluble. Warm water was added to permanent opalescence and the mixture was allowed to crystallize. The osazone was thus obtained as a felt of lemon-yellow needles.

It had the following composition.

4.971 mg. substance: 10.843 mg.  $\text{CO}_2$  and 2.910 mg.  $\text{H}_2\text{O}$

5.075 " " : 0.631 cc. N ( $29^\circ$  and 756 mm.)

$\text{C}_{20}\text{H}_{28}\text{O}_5\text{N}_4$ . Calculated. C 59.66, H 6.51, N 13.93

Mol. wt. 402.3. Found. " 59.48, " 6.55, " 14.03

The osazone melted sharply at  $160^\circ$  to a clear liquid which decomposed at  $198$ – $200^\circ$  (not corrected). The rotation of the osazone in pyridine + absolute alcohol (2:3 by volume) was

$$[\alpha]_D^{20} = \frac{+0.38^\circ \times 100}{1 \times 3.5} = +10.9^\circ \text{ initial}$$

and 
$$[\alpha]_D^{20} = \frac{+0.70^\circ \times 100}{1 \times 3.5} = +20.0^\circ \text{ at equilibrium}$$

### Procedure

The sugars were dried over phosphorus pentoxide under reduced pressure to approximately constant weight and the moisture was then determined. It was never greater than 0.4 per cent and the amounts taken were corrected to a dry basis. The calculated quantity of the dry substance was weighed into a volumetric flask and dissolved in anhydrous methyl alcohol.<sup>13</sup> The calculated volume of a standardized solution of dry hydrogen chloride in anhydrous methyl alcohol was added with a pipette and the mixture was immediately diluted to volume and well shaken to insure complete mixing.

Experiments were made at room temperature and at 76°. In the latter case portions of the mixture were sealed in Pyrex test-tubes which were immersed in a bath of boiling carbon tetrachloride for the required intervals of time, then removed, and cooled immediately in ice.

### Analytical

The entire analytical procedure has been previously described.<sup>6</sup> Two methods were employed, the Hagedorn-Jensen,<sup>14</sup> as modified by Hanes,<sup>15</sup> and the Willstätter<sup>16</sup> hypiodite oxidation, on both a macro and micro scale. The samples were removed from the reaction mixtures and neutralized, and were then analyzed before and after acid hydrolysis. Methyl alcohol was found to decrease the hydrolysis of the  $\gamma$ -glucosides and was therefore removed by distillation under reduced pressure.

For the methylated sugars employed in this work as well as for the glucoheptose, the Hagedorn-Jensen reduction values were not available and they were therefore determined. The results are given in Table II. For glucose the values of Sobotka and Reiner<sup>17</sup> were used.

In making the calculations it was necessary to know the effect

<sup>13</sup> In the case of glucoheptose, its low solubility made it necessary to shake the solid with the methyl alcoholic hydrogen chloride; the values, therefore, are only approximate.

<sup>14</sup> Hagedorn, H. C., and Jensen, B. N., *Biochem. Z.*, **135**, 46 (1923).

<sup>15</sup> Hanes, C. S., *Biochem. J.*, **23**, 99 (1929).

<sup>16</sup> Willstätter, R., and Schudel, G., *Ber. chem. Ges.*, **51**, 780 (1918).

<sup>17</sup> Sobotka, H., and Reiner, M., *Biochem. J.*, **24**, 394 (1930).



of the acid treatment upon the reducing value of the sugars as this had been found in other cases to be quite appreciable. For this reason known amounts of the sugars were analyzed before

TABLE II  
*Reduction Value by Hagedorn-Jensen (Hanes) Method. Cc. 0.01 N Thiosulfate*

Sugar	$\alpha$ -Glucoheptose	2-Methylglucose	3-Methylglucose	4-Methyl- $\alpha$ -glucoheptose
<i>mg.</i>				
1.0	2.29	1.62	3.18	1.72
2.0	4.52	4.32	6.58	3.49
3.0	6.76	7.38	9.94	5.37
4.0	9.08	10.42		7.29

TABLE III  
*Analytical Factors*

Sugar	Present	0.01 N thiosulfate	Found		Hydrolysis with HCl*	Analytical method†
	<i>mg.</i>	<i>cc.</i>	<i>mg.</i>	<i>per cent</i>		
Glucose	3.0	9.06	3.03	101	None	H.-J.-H.
	5.0	5.45	4.91	98	0.1 N	W.
$\alpha$ -Glucoheptose	3.0			100	None	H.-J.-H.
	3.0	7.35	3.23	108	0.05 N	"
2-Methylglucose	3.0			100	None	"
	3.0	7.33	2.99	100	0.05 N	"
3-Methylglucose	3.0			100	None	"
	3.0	9.74	2.95	98	0.1 N	"
4-Methyl- $\alpha$ -glucoheptose	3.0			100	None	"
	3.0	5.52	3.10	103	0.05 N	"
Tetramethylglucopyranose	63.8	53.4	63.0	98	None	W.
	65.0	53.9	63.6	98	0.1 N	"
Tetramethylglucofuranose	63.9	46.1	54.4	85	None	"
	35.2	25.2	29.8	85	0.1 N	"

\* At 100° for 10 minutes.

† H.-J.-H. represents the Hanes modification of the Hagedorn-Jensen method; W., the Willstätter method.

and after acid treatment. The factors thus found are given in Table III. The fact that the tetramethylglucofuranose gives but 85 per cent of the theoretical reducing value by the hypiodite method either before or after hydrolysis is taken to mean that this product, due to its method of preparation, contains 15 per cent of glucopyranoside. This would not affect the results on glucoside formation but would lead to fictitious values for the specific rotations during glucoside formation.

The method of calculating the proportions of pyranoside and furanoside has been discussed in detail<sup>6</sup> and need not be repeated here. The stability of the pyranosides towards acid hydrolysis

TABLE IV  
*Stability of Normal Glycosides*

Sugar	0.01 N thiosulfate		Sugar found		Sugar present	Free sugar		Hydroly- sis with HCl at 100° for 10 min.
	Before hydroly- sis	After hydroly- sis	Before hydroly- sis	After hydroly- sis		Before hydroly- sis	After hydroly- sis	
	cc.	cc.	mg.	mg.	mg.	per cent	per cent	n
Glucose	0.06	0.07	0.02	0.16	3.10	1	5	0.1
2-Methylglucose	0.15	0.64	0.11	0.46	3.34	3	14	0.1
		0.29		0.21	3.34		6	0.05
3-Methylglucose	0.09	0.53	0.03	0.16	3.34	1	5	0.1
4-Methyl- $\alpha$ -gluco- heptose	0.09	0.72	0.05	0.42	3.86	2	11	0.1
		0.36		0.21	3.86		5	0.05
$\alpha$ -Glucoheptose	0.16	1.86	0.07	0.81	3.61	2	22	0.1
		1.24		0.54	3.61		15	0.05

was determined as it is needed for the calculations. The data given in Table IV include, in some cases, two concentrations of acid. The one actually employed in the analyses is given in Table III.

#### *Structure of Glucoheptosides*

*Glycoside from 4-Methyl- $\alpha$ -Glucoheptose*—In order to determine the ring form of the glycoside in the case of the 4-methyl- $\alpha$ -glucoheptose, the reaction was discontinued after 48 hours and the hydrogen chloride was removed with silver oxide. The mixture was made faintly alkaline with a few drops of a solution of barium hydroxide in methyl alcohol, and concentrated under reduced pressure

to a syrup. This was dissolved in methyl iodide and methyl alcohol and methylated by the Purdie method. The product was soluble in methyl iodide alone and was remethylated in this solvent. After distillation of the product, the analysis corresponded fairly well to that of a pentamethylmethylheptoside.

3.056 mg. substance: 5.290 mg.  $\text{CO}_2$  and 2.405 mg.  $\text{H}_2\text{O}$   
 5.790 " " : 26.975 "  $\text{AgI}$   
 $\text{C}_{15}\text{H}_{28}\text{O}_7$ . Calculated. C 53.02, H 8.91,  $\text{OCH}_3$  63.22  
 Mol. wt. 294.2. Found. " 52.82, " 8.80, " 61.49

This material was hydrolyzed with 1.7 N hydrochloric acid for 9 hours at  $85^\circ$  and then for 2 hours on the steam bath. The neutralized solution was extracted with chloroform; the extract was dried, concentrated to a syrup, and distilled under greatly reduced pressure. The product crystallized on standing and was recrystallized from a mixture of pentane and ether (4:1). The analysis corresponded to a pentamethylheptose.

4.350 mg. substance: 8.114 mg.  $\text{CO}_2$  and 3.340 mg.  $\text{H}_2\text{O}$   
 5.264 " " : 22.210 "  $\text{AgI}$   
 $\text{C}_{15}\text{H}_{28}\text{O}_7$ . Calculated. C 51.39, H 8.63,  $\text{OCH}_3$  55.32  
 Mol. wt. 280.2. Found. " 50.86, " 8.59, " 55.68

The specific rotation, in water, was  $-8.0^\circ$  initial, and  $[\alpha]_D^{20} = -0.89^\circ \times 100 = -44.5^\circ$  after 48 hours (equilibrium). The substance melted unsharply at about  $40^\circ$ , partly resolidified, and remelted at about  $80^\circ$  to a clear liquid. Haworth<sup>5</sup> gives  $-62.5^\circ$  and  $-42.5^\circ$  for the initial and final rotations of the  $\beta$  form and  $84^\circ$  as the melting point.

For final identification, the pentamethylheptose was oxidized with barium hypoiodite, the oxidation product was extracted from the acidified solution with ether, and the dried extract was concentrated under reduced pressure to a syrup. This crystallized after standing overnight and was recrystallized from a mixture of ethyl acetate and pentane.

The analysis of the product corresponded to a pentamethylheptonic lactone.

4.087 mg. substance: 7.695 mg.  $\text{CO}_2$  and 2.990 mg.  $\text{H}_2\text{O}$   
 4.684 " " : 19.900 "  $\text{AgI}$   
 $\text{C}_{15}\text{H}_{28}\text{O}_7$ . Calculated. C 51.76, H 7.97,  $\text{OCH}_3$  55.71  
 Mol. wt. 278.2. Found. " 51.34, " 8.18, " 56.08

The product melted at 84–85° and had a specific rotation, in water, of +45.5° initial, and  $[\alpha]_D^{20} = \frac{+0.20^\circ \times 100}{2 \times 2.0} = +5.0^\circ$  after 48 hours (equilibrium). Haworth gives 83° for the melting point of 2, 3, 4, 6, 7-pentamethyl- $\alpha$ -glucoheptonic lactone, +40° for the initial rotation, and +9.4° for that at equilibrium (after 38 hours).

It is, therefore, evident that the glycoside formed from 4-methylglucoheptose at room temperature after 48 hours is the pyranoside.

*Lactone from  $\alpha$ -Glucoheptose*— $\alpha$ -Glucoheptonic lactone, prepared from glucose through the cyanhydrin, was methylated by the Purdie method, and after isolation was remethylated by the same method. After distillation the higher boiling fraction had a methoxyl content which was too high for the pentamethylheptonic lactone (due to the presence of methylated ester), whereas that of the lower boiling fraction was nearly theoretical. After occasional scratching with a glass rod over a period of several days the lower boiling fraction crystallized. Recrystallization from ethyl acetate and pentane gave a product the analysis of which corresponded to a pentamethylheptonic lactone.

3.722 mg. substance: 7.070 mg. CO<sub>2</sub> and 2.719 mg. H<sub>2</sub>O

5.661 " " : 23.462 " AgI

C<sub>13</sub>H<sub>22</sub>O<sub>7</sub>. Calculated. C 51.76, H 7.97, OCH<sub>3</sub> 55.71

Mol. wt. 278.2 Found. " 51.79, " 8.16, " 54.70

The rotation, in water, was -10.8° initial, and -9.0° after 24 hours, but the equilibrium was not reached in 10 days (-2.0°). For confirmation the lactone was converted to the salt with excess sodium hydroxide and the acid was liberated with the exact equivalent of hydrochloric acid. The specific rotation was +20.0° initial, and  $[\alpha]_D^{20} = \frac{+0.15^\circ \times 100}{1 \times 2.0} = +7.5^\circ$  at apparent equilibrium (after 30 days).



# INTESTINAL NUCLEOTIDASE AND POLYNUCLEOTIDASE

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## INTRODUCTION

The gastrointestinal secretions of the dog<sup>1,2</sup> contain the most effective agent for the hydrolysis of thymus nucleic acid into its component nucleotides that is known at the present time. However, the efficiency of even this agent is very low, so that the weight of the active material required for maximum hydrolysis which is about 75 per cent of the theory is equal to 60 per cent of the nucleic acid to be hydrolyzed. Thus the hydrolysate contains much extraneous material which renders the isolation of the small amounts of pure nucleosides difficult. On the other hand, this same agent is of very much greater efficiency for the hydrolysis of a number of simple esters of phosphoric acid. The desirability of increasing the efficiency of the material to be used for nucleic acid hydrolyses is self-evident but attempts to accomplish this end were unsuccessful.

It is obvious that the difference in efficiency may be due to one of two causes, either to the greater stability of nucleic acids compared with the simpler phosphoric esters, or to the presence in the gastrointestinal secretions of two different agents, one a more or less non-specific phosphatase and the other a specific agent adapted to nucleic acids only. Evidence points to the latter case and for simplicity the agent hydrolyzing the simple phosphates including the mononucleotides, that is, the non-specific phosphatase, is referred to as *nucleotidase* and the agent required for the hydrolysis of nucleic acids is referred to as *polynucleotidase*.

<sup>1</sup> Levene, P. A., and London, E. S., *J. Biol. Chem.*, **81**, 711 (1929); **83**, 793 (1929).

<sup>2</sup> Levene, P. A., and Dillon, R. T., *J. Biol. Chem.*, **88**, 753 (1930).

In the first communication of this series<sup>2</sup> an observation was reported which suggested the presence of polynucleotidase in the gastrointestinal secretions, in addition to the non-specific phosphatase. Thus, a sample of the active material which possessed a nucleotidase activity much lower than normal was obtained, yet it had a higher polynucleotidase activity than usual. A number of similar observations showing that the two activities do not run parallel were encountered in the course of the present work. A summary of them is given in Table I.

Additional evidence to the same effect was found in the following observations.

1. The dry active material taken up in water dissolves incompletely, leaving appreciable amounts of solid in suspension. This

TABLE I  
*Nucleotidase and Polynucleotidase Activity. Variation with Enzyme Sample*

Sample	Activity coefficients		Ratio of coefficients nucleotidase to polynucleotidase
	Nucleotidase	Polynucleotidase	
	<i>units per mg.</i>	<i>units per mg.</i>	
a	1.01	0.029	34.8
b	0.75	0.113	6.6
c	0.99	0.128	7.7
d	0.43	0.112	3.8
e	0.55	0.114	4.8
f	1.18	0.096	12.3

suspended material when separated from the solution by centrifuging contains a higher proportion of nucleotidase than the original sample. In Table II the ratio of nucleotidase to polynucleotidase for the insoluble part is seen to be 20:1; whereas, for the original material it was 8:1. Acetone precipitation of the active material left after removal of the insoluble part gives a substance with a ratio of about 8:1.

2. The optimal activity of the nucleotidase<sup>3</sup> is at a pH > 11

<sup>3</sup> Schmidt (Schmidt, G., *Klin. Woch.*, 10, 165 (1931)) reports two pH optima for the action of liver nucleotidase on guanylic acid; namely, at pH 5.0 to 6.0 and at 9.0. The enzyme was reported to be specific for this nucleotide. Most phosphatases are reported to have their optimal activity at pH 9.0.

while that of the polynucleotidase is at pH = 8.5. Fig. 1 shows the activity-pH curves for the different substrates.

3. Fluoride and arsenate ions affect the nucleotidase and polynucleotidase activities somewhat differently. The polynucleotidase activity appears to be inhibited by fluoride ion to a much greater degree than is nucleotidase.<sup>4</sup> The difference with regard to arsenate ion is less evident, both enzymes being markedly inhibited. The results are given in Table III.

These observations encouraged an attempt to separate the two enzymes. The methods resorted to for this purpose were: (1) dialysis, (2) fractional precipitation, and (3) adsorption with subsequent elution. Thus far the separation has not been

TABLE II  
*Insoluble Enzyme Fraction. Variation with pH*

Sample	pH	Weight	Activity coefficients		Ratio of coefficients nucleotidase to polynucleotidase	Total activity	
			Nucleotidase	Polynucleotidase		Nucleotidase	Polynucleotidase
		gm.	units per mg.	units per mg.		units	units
Original enzyme		3.00	0.80	0.100	8.0	2.40	0.300
Insoluble fraction	4.5	0.22	1.07			0.24	
" "	7.0	0.88	1.45	0.075	19.3	1.28	0.066
" "	8.5	0.93	1.38	0.071	19.4	1.28	0.066
" "	8.5	0.90	1.38			1.24	

accomplished but the efforts toward this end have furnished further evidence in favor of the existence of two different phosphatase agents.

The results of *dialysis* experiments are given in Table IV, from which it can be seen that the nucleotidase and polynucleotidase activities are probably affected by dialysis in different ways. In one experiment the nucleotidase activity per unit of weight decreased whereas that of the polynucleotidase increased; in the second experiment both activities increased somewhat, but polynucleotidase increased to a greater degree.

<sup>4</sup> On the other hand, Schmidt<sup>3</sup> reports that fluoride ion at a concentration as low as 0.001 M completely inhibits the action of liver nucleotidase on guanylic acid.



*Fractional precipitation with acetone leads to materials in which the ratios of nucleotidase to polynucleotidase are increased in*

TABLE III  
*Nucleotidase and Polynucleotidase Activity. Effect of Fluoride and Arsenate Ion*

Added substance	Concentration	Hydrolysis			
		Glycerol phosphate	Adenylic acid	Guanylic acid	Thymus nucleic acid
	M	per cent	per cent	per cent	per cent
Control		40.6	39.6	38.2	77.0*
Fluoride ion	0.001	39.2			
" "	0.005	40.4	38.0	38.2	
" "	0.010	40.6			75.8
" "	0.050	38.8			
" "	0.100		39.6	39.0	
" "	0.133	38.6			23.0
Arsenate "	0.005	15.0	10.4	9.4	
" "	0.010	10.2			44.7
" "	0.100	4.8	3.0	3.2	
" "	0.133				3.5

\* The nucleic acid control is in the presence of veronal buffer.

TABLE IV  
*Dialysis of Intestinal Secretions*

Samples and treatment	Acetone precipitate obtained	Activity coefficients		Total activity	
		Nucleotidase	Poly-nucleotidase	Nucleotidase	Poly-nucleotidase
	gm.	units per mg.	units per mg.	units	units
Intestinal secretion (original)					
100 cc. undialyzed	0.30	0.55	0.114	0.165	0.0342
100 " dialyzed	0.14	0.40	0.124	0.056	0.0174
Intestinal secretion concentrated 7-fold*					
100 cc. undialyzed	1.50	1.18	0.096	1.77	0.144
100 " dialyzed	0.96	1.22	0.113	1.17	0.109

\* Unfortunately, this 7-fold concentrated solution was not prepared from the preceding original secretions. Hence, a comparison between the dilute and concentrated secretions in this table is not possible.

the first fractions of a series, but in the final fraction the proportion of polynucleotidase is much higher than in the original material. Unfortunately, this method of treatment involves a considerable loss of total activity (Table V).

The results of the *adsorption* experiments are given in Table VI. Silica gel, aluminum hydroxide,<sup>5</sup> colloidal iron, and calcium phosphate<sup>6</sup> were used. All of these substances adsorb preferen-

TABLE V  
*Nucleotidase and Polynucleotidase Activity. Acetone Precipitation of Enzyme*

Purification	Sample	Weight gm.	Activity coefficients		Ratio of coefficients nucleotidase to polynucleotidase	Total activity	
			Nucleotidase units per mg.	Polynucleotidase units per mg.		Nucleotidase units	Polynucleotidase units
Repeated acetone precipitation	Original	1.00	0.85			0.85	
	1st reprecipitation	0.87	1.00			0.87	
	2nd "	0.25	1.27			0.32	
	3rd "	0.10	1.32			0.13	
Fractional acetone precipitation	Original	4.0	0.51	0.108	4.7	2.04	0.432
	Insoluble + ppt. with $\frac{1}{2}$ volume acetone	1.5	0.95	0.092	10.3	1.42	0.138
	Ppt. with 1 volume acetone	0.6	0.72	0.121	5.9	0.43	0.073
	Ppt. with 10 volumes acetone	0.3	0.08	0.053	1.5	0.02	0.016

tially the polynucleotidase leaving a solution in which the ratio of nucleotidase to polynucleotidase is increased. Had it been possible to elute the polynucleotidase from the adsorbent in active form the adsorption might have led to a considerable concentration

<sup>5</sup> Aluminum hydroxide  $C_7$  was used. For its preparation see Willstätter, R., Kraut, H., and Erbacher, O., *Ber. chem. Ges.*, **58**, 2448 (1925).

<sup>6</sup> Both tribasic and dibasic phosphate were used.  $Ca_3(PO_4)_2$  is precipitated in alkaline solution and  $CaHPO_4$  in neutral solution.

of this enzyme. Unfortunately, the material seems to be inactivated on the surface of the adsorbent.

*Nature of Nucleotidase and of Polynucleotidase*—It has already been stated that the hydrolysis of mononucleotides proceeds at the

TABLE VI  
*Adsorption of Polynucleotidase Activity*

Enzyme after adsorption with	Adsorption pH	Weight	Activity coefficients		Ratio of coefficients nucleotidase to polynucleotidase	Total activity	
			Nucleotidase	Polynucleotidase		Nucleotidase	Polynucleotidase
		gm.	units per mg.	units per mg.		units	units
	Original	3.0	0.80	0.100	8.0	2.40	0.300
Silica gel, 4 times	4.5	1.8	0.70	0.075	9.3	1.26	0.135
“ “ 4 “	7.0	1.1	0.75	0.081	9.3	0.82	0.089
“ “ 4 “	8.6	1.3	0.65	0.034	19.1	0.85	0.043
“ “ 4 “ *	8.6	0.9	0.80	0.037	21.9	0.72	0.033
“ “ 4 “	8.6	1.3	0.85	0.034	26.2	1.11	0.044
“ “ 2 “	8.6	1.5	0.80	0.039	20.4	1.20	0.059
Al(OH) <sub>3</sub>	8.6	1.0	0.72	0.038	18.9	0.72	0.038
Fe(OH) <sub>3</sub>	6.5	1.3	0.72	0.043	16.7	0.94	0.056
Ca <sub>3</sub> (PO <sub>4</sub> ) <sub>2</sub>	8.6	0.4	0.50	0.053	9.5	0.20	0.021
CaHPO <sub>4</sub>	6.5	0.9	0.80	0.082	9.8	0.72	0.074

\* Adenylic acid hydrolysis with this sample gave results identical with the original enzyme. This confirms the previous observation that glycerol phosphate and nucleotides are parallel in their hydrolysis rates even though the nucleic acid hydrolysis is low.

same rate as that of glycerol phosphate or of other simple esters of phosphoric acid and hence the agent hydrolyzing mononucleotides, that is nucleotidase, may be regarded as a simple phosphatase. The nature of the polynucleotidase is not so readily disclosed. It

is possible that the rôle of this agent is to hydrolyze the polynucleotides to mononucleotides which are then dephosphorylated by the non-specific phosphatase. On the other hand, it may play the part of a specific phosphatase adapted to polynucleotides only. A decision could be reached easily if an accurate method for the estimation of mononucleotides were available. In the absence of such, the polynucleotidase activity was measured by the rate of phosphoric acid cleavage from polynucleotides by a material which contained in addition to this specific agent, the non-specific phosphatase. Hence an answer to the true rôle of the polynucleotidase must be postponed at least until a method for the quantitative estimation of mononucleotides is developed.

#### EXPERIMENTAL

*I. The Enzyme*—Nucleotidase-polynucleotidase prepared by acetone precipitation of the intestinal secretions of dogs served as the source of enzyme material. Some of the general properties of these intestinal secretions and of the acetone-precipitated enzyme have been described in the previous paper.<sup>2</sup> Therein it was shown that the enzyme was a potent non-specific nucleotidase hydrolyzing with almost equal rapidity glycerol phosphate, natural and synthetic hexosephosphates, and nucleotides. However, the hydrolysis of nucleic acids to phosphoric acid and nucleosides was not so rapid, yeast and thymus nucleic acid being hydrolyzed, respectively, only one-fifteenth and one-twentieth as fast as glycerol phosphate.

The enzyme is quite stable. The dry samples apparently keep indefinitely with little or no loss in activity. The original intestinal secretions have been allowed to stand as long as 1 month at 25° without any loss in their nucleotidase activity while the polynucleotidase activity had decreased only slightly. Samples left standing in solution were protected with a small amount of toluene. The amount of acetone-precipitable material decreased somewhat during this interval which resulted in a loss of the total activity recovered since the activity coefficients were no higher than those of samples from fresh intestinal secretions.

The acetone-precipitated enzyme is readily suspended in water giving a solution with a pH of 6.5 to 7.0. About one-third of the material of a 10 per cent solution is easily centrifuged off, giving a

nearly transparent yellow solution. This clear solution continues to coagulate very slowly on standing. From this solution the enzyme is precipitated slightly at a pH of 4.3 and completely at a pH of 3.6 to 3.8. Solutions adjusted to a pH of 4.3 are slowly inactivated while at a slightly lower pH, inactivation is rapid and complete. On the alkaline side the enzyme is evidently stable up to a pH of 11.0. Some precipitation of the enzyme material occurs at a pH of 8.5 which removes only a small fraction of the activity. Inactivation, however, does not take place as in the case of precipitation on the acid side, and merely resuspending the precipitate restores the original activity.

For this work the enzyme solutions were prepared by suspending the acetone-precipitated material in water or substrate solution at the desired concentration. In a few cases the original intestinal secretions were used. For glycerol phosphate hydrolyses these secretions were used directly while for the nucleic acid hydrolyses they were first concentrated about 7-fold. This was done rapidly at low temperature and under reduced pressure. For example, 2700 cc. of secretions were concentrated to 400 cc. in 3 hours at a temperature of less than 10°. This increase in concentration of enzyme was sufficient so that the solution could be used directly by mixing with the substrate solution.

*II. Units of Activity*—In the previous communication only the unit of nucleotidase activity was introduced. In the present considerations the polynucleotidase activity unit is also essential. The units for these two activities which were adopted are defined as follows:

The unit of *nucleotidase (phosphatase) activity* is the amount of enzyme capable of liberating from 1.0 cc. of a standard glycerol phosphate substrate (0.161 M, 5.0 mg. of P per cc.) 1.0 mg. of phosphorus (as orthophosphate ion) in the course of the 1st hour of hydrolysis. This corresponds to 20 per cent hydrolysis of the total hydrolyzable phosphorus. Since this value is so close to the beginning of the hydrolysis the per cent hydrolyses are proportional to the initial rates of hydrolysis. The latter have been shown to be proportional to the enzyme concentrations.<sup>2</sup>

The unit of *polynucleotidase activity* is the amount of enzyme capable of liberating from 1.0 cc. of a standard thymus nucleic acid substrate (0.0093 M, 1.14 mg. of P per cc.) 0.57 mg. of phos-

phorus in the course of the first 6 hours of hydrolysis. This corresponds to 50 per cent hydrolysis of the hydrolyzable phosphorus.

However, this value is so far advanced in the hydrolysis that it is not exactly proportional to the initial rate of hydrolysis. But within a very limited region close to this amount of hydrolysis the values will be indicative of the enzyme activity. Outside of this range the value can be considered only as an approximation.<sup>7</sup> The values of activity greater than unity are accordingly too small and those less than unity, too large.

Both activity units are based on the hydrolysis at a pH of 8.6 to 8.7 and at a temperature of 30°. The concentrations of dry acetone-precipitated enzyme used in these assays were 2.0 mg. and 12.0 mg. per cc. for the nucleotidase and polynucleotidase determinations, respectively. The corresponding activity coefficients, in activity units per mg. of enzyme, are then given by dividing the units of enzyme by 2.0 and 12.0. The total activity is the product of this coefficient and the total weight of enzyme.<sup>8</sup>

*III. Hydrolyses. General Methods*—The method of preparing the substrates, of performing the hydrolyses, and of analyzing the hydrolysates for phosphate ion has been discussed in the first paper of this series.<sup>2</sup> Essential points to be recalled or added to the previous description include the following.

For the glycerol phosphate and nucleotide hydrolyses no buffers were added to the substrate since the original pH of the solution was maintained throughout complete hydrolysis. In the case of nucleic acid a veronal buffer<sup>9</sup> was used (1.0 cc. of 0.1 M veronal buffer solution (pH 8.6) to 2.0 cc. of nucleic acid substrate solution) except in certain experiments where previously prepared solutions of the enzyme were used and conditions did not permit

<sup>7</sup> In fact, owing to the complicated nature of the hydrolyses involved, in particular the fact that the nature of nucleic acid probably allows 25 per cent of the phosphorus to be hydrolyzed directly from this substrate by a simple nucleotidase, the true significance of hydrolysis of half of that taken as the unit (that is, 25 per cent hydrolysis) is questionable in regard to its relation to the polynucleotidase activity of the enzyme sample.

<sup>8</sup> In order to avoid large numbers, the total activities reported are the product of the activity coefficients, in units per mg., and the weight in gm. (instead of mg.).

<sup>9</sup> Michaelis, L., *J. Biol. Chem.*, **87**, 33 (1930).

the additional dilution necessary to introduce the buffer. However, in all of the nucleic acid hydrolyses the pH had to be adjusted slightly with alkali at various intervals in order to keep the variation less than  $\pm 0.2$  of a pH. The first amounts of phosphate ion liberated gave the solutions additional buffering capacity so that the variations then became quite small.

The extent of hydrolysis was determined by measuring the increase in the concentration of inorganic phosphate ion. The phosphate determinations were made colorimetrically by the method of Kuttner and Cohen.<sup>10</sup> Details of the analyses differed according to the type of substrate and have been described previously.<sup>2</sup> The phosphorus determinations were corrected for the initial (blank) determinations of the inorganic phosphorus. These blank corrections amounted to approximately 0.05 mg., 0.08 mg., and 0.25 to 0.35 mg. of phosphorus per cc. of hydrolysate for the glycerol phosphate, nucleotide, and nucleic acid substrates, respectively. In the case of initial determinations and of low hydrolysis values involving precipitation of the phosphate, standard amounts of phosphate ion were added to the samples for analysis so as to insure comparable precipitation of the magnesium ammonium phosphate.

*IV. Influence of pH on Activities*—The activity of the enzyme at different pH values was determined for glycerol phosphate, adenylic acid, and thymus nucleic acid. Presumably these substrates may be taken as typical of three classes of organic phosphates; namely, simple organic sugar phosphates, nucleotides, and nucleic acid. The substrate solutions were adjusted to the indicated pH values and with the exception of the nucleic acid solutions with initial values greater than 7.5 the hydrolyzing solutions did not change in pH. Those that changed were readjusted so that the variations were less than  $\pm 0.2$  of a pH. The concentrations of enzyme and substrate were the same as indicated for the activity assays. The lengths of the periods of hydrolysis were 1.5, 2.0, and 5.0 hours for the glycerol phosphate, adenylic acid, and thymus nucleic acid, respectively. The hydrolyses are summarized in Table VII which also indicates typical phosphate determinations as made throughout the work. The per cent

<sup>10</sup> Kuttner, T., and Cohen, H. R., *J. Biol. Chem.*, **75**, 517 (1927).

hydrolyses of the three substrates at the various pH values are plotted in Fig. 1.

*V. Inhibition Effects*—The original enzyme secretions contain a substance which inhibits the hydrolysis of both glycerol phosphate and nucleic acid. It is obviously impossible to tell whether the effect on the nucleic acid hydrolysis results from the inhibition

TABLE VII  
*Nucleotidase and Polynucleotidase Activity. Variation of Hydrolysis with pH*

Substrate	pH	Phosphorus (as $\text{PO}_4^{\equiv}$ )		
		Found		Increase during hydrolysis
		Initial	After hydrolysis	
		mg. P per cc.	mg. P per cc.	mg. P per cc.
Glycerol phosphate	5.0	0.04	0.04	0.00
	5.5	0.05	0.05	0.00
	6.2		0.07	0.03
	6.8	0.04	0.27	0.23
	7.5		0.98	0.94
	8.5	0.04	2.57	2.53
	9.0		2.94	2.90
	9.5		3.09	3.05
	10.0	0.05	3.24	3.19
	11.0		3.44	3.40
Adenylic acid	5.5	0.08	0.20	0.12
	6.8		0.40	0.32
	7.6		1.32	1.24
	8.5	0.07	2.04	1.96
	9.6		2.92	2.84
Thymus nucleic acid	6.8	0.366	0.612	0.246
	7.6		0.718	0.352
	8.5	0.366	0.966	0.600
	9.4		0.612	0.246

of both the polynucleotidase and nucleotidase or from the inhibition of the latter alone.

Inhibition of the glycerol phosphate hydrolysis was demonstrated in the previous paper. In the case of nucleic acid, it is shown by the fact that hydrolysis directly with the 7-fold concentrated solution increases from 35 per cent to 50 per cent by dialysis for 36 hours. The inhibitor is evidently not completely removed



by a single acetone precipitation because the total nucleotidase activity recovered (for example, in simple reprecipitation with acetone or on adsorption with silica gel or aluminum hydroxide) was frequently slightly greater than that started with in spite of the loss in material.

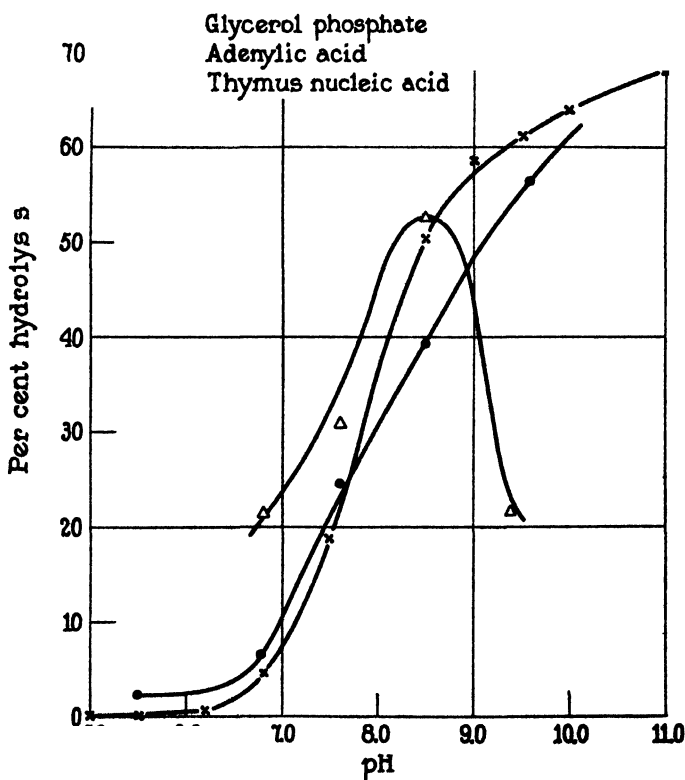


FIG. 1. Nucleotidase and polynucleotidase activity. Variation with pH.

The inhibition of a few buffer solutions on the hydrolyses has been indicated in the previous work. The present work adds to these results the effect of arsenate and fluoride ions at various concentrations. The data are given in Table III. Fig. 2 indi-

cates the relative hydrolysis of glycerol phosphate, adenylic, guanylic, and thymus nucleic acid with respect to the concentration of added arsenate on fluoride ion. In reality, the differences in the effect on the two types of activity are more marked in the case of fluoride ion and less marked in the case of arsenate ion than the curves of Fig. 2 indicate, since a higher concentration of enzyme was used in the case of nucleic acid than for the other substrates.

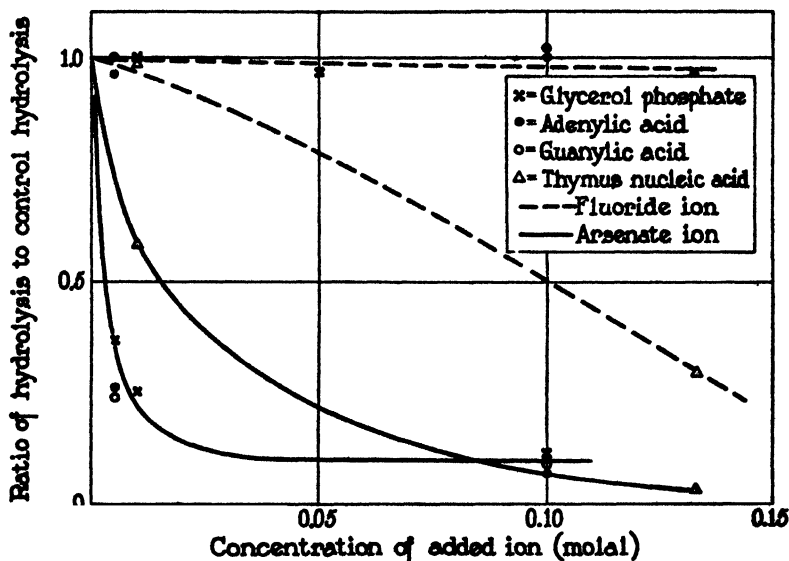


FIG. 2. Nucleotidase and polynucleotidase activity. Effect of arsenate and fluoride ions.

*VI. Dialyses*—Two solutions were dialyzed, one the original intestinal secretions and the other the intestinal secretions after a 7-fold concentration. They were dialyzed against distilled water in 1 inch Visking sausage casings.<sup>11</sup> The bottom of the piece of casing was closed with a solid rubber stopper held in place by tight rubber bands and in the top was a 1-hole stopper by which the filled tube was suspended in water. Both solutions increased

<sup>11</sup> Tubing suitable for dialyses may be obtained from the Visking Corporation, Chicago, in various sizes and lengths. Before use it must be carefully tested for leaks.

in volume during the dialyses, the concentrated solution almost doubling in volume. The dialysates were free of chloride ion after 24 and 48 hours for the dilute and concentrated solutions, respectively, after which time the dialyses were stopped. Acetone-precipitated samples of both dilute and concentrated solutions, before and after dialysis, were prepared in the usual manner. The most significant result was the large decrease in acetone-precipitable material after dialysis, the yield from equivalent amounts of solution decreasing to approximately one-half in the two solutions dialyzed (Table IV). A comparison of the dialyzed and undialyzed samples shows that the nucleotidase activity coefficients were practically the same in both cases. The polynucleotidase values were in both cases higher in the dialyzed samples. The differences between the dialyzed and undialyzed samples were, however, not large and in view of the large loss of material on dialysis the total activities were lowered appreciably. Hydrolyses performed directly on the dialyzing solutions indicated that the substance inhibiting the hydrolyses was partially dialyzable.

*VII. Fractional Precipitation with Acetone*—For reprecipitation of the enzyme with acetone 10 per cent solutions were usually prepared. These were sometimes used directly but in most cases were used after removing the readily centrifuged material. Table II shows the variation of this insoluble material with pH and the activities of these fractions. For precipitation of the remaining material with acetone the supernatant liquids were usually readjusted to a pH of 6.8 to 7.2 since this value seemed to be most satisfactory for yielding a fine, easily dried enzyme. The activity coefficients of the acetone-precipitated material from the supernatant liquid of these centrifuged solutions were always about the same as the original acetone precipitates.

Reprecipitation of the enzyme with acetone was carried out in the same manner as for the original precipitation. The aqueous solution of the enzyme was poured rapidly with continuous stirring into the acetone (8 to 10 volumes), allowed to settle, and was resuspended once again by stirring. After the second sedimentation, the supernatant liquid was decanted and the precipitate was centrifuged lightly. Too long centrifuging of the precipitate at this stage packed the solid so that it was difficult to resuspend in acetone for washing and resulted in material which did not dry

readily. The solid was next washed once with acetone and twice with anhydrous ether, centrifuged between washings, and was then put into a vacuum desiccator over phosphorus pentoxide and paraffin. The whole operation was completed in 15 to 20 minutes. Repeated precipitation of the enzyme from a 4 per cent solution with 10 volumes of acetone resulted in a large loss of the activity even though the activity coefficient was increased somewhat. The greatest loss of material occurred during the second and third reprecipitations at which time it had become almost snow-white and dissolved in water, giving a practically colorless transparent solution with no evidence of insoluble material. The results of this reprecipitation are shown in Table V.

Fractional precipitation of the enzyme was not successful. While invariably 75 to 85 per cent of the enzyme could be recovered from a 10 per cent solution of once acetone-precipitated material by precipitation with 10 volumes of acetone, only 50 per cent could be recovered if the precipitation were done fractionally. Not only was there a large loss of material but the activity coefficients were lowered (Table V).

*VIII. Adsorption Experiments*—The adsorption of the enzyme on various reagents was investigated. *Cholesterol* was used on the original secretions. It was prepared in a very finely divided state in the following manner. To 150 cc. of glycerol were added 11 gm. of pure cholesterol. The mixture was heated to 150° at which temperature the cholesterol had melted and the mixture had become homogeneous. It was then allowed to cool very slowly, stirring vigorously all the while. At 100°, 10 cc. of hot water were added and the cooling and stirring continued until room temperature was reached. When cold, the mixture was a stiff snow-white paste which consisted of very minute crystals of cholesterol surrounded by an immense number of small air bubbles.

This paste was mixed with the enzyme solution by stirring for 30 minutes. The finely divided cholesterol was then removed by filtration through a Buchner funnel covered with a thin layer of asbestos. This asbestos layer was poured into place on top of a piece of thin silk. The top of the asbestos was also protected with silk. Filtration of the solution was effected in this way in a few hours, whereas otherwise it was almost impossible to accomplish. The enzyme was then precipitated from the filtrate in the usual

way. The activity coefficients and total activity were practically identical with the enzyme precipitated without this treatment.

*Silica gel*, *aluminum hydroxide*, and *calcium phosphate* were found to adsorb the polynucleotidase agent in the alkaline region while colloidal *ferric hydroxide* adsorbed it in slightly acid medium. Centrifuged solutions of the enzyme were treated with the adsorbents as follows: The supernatant liquid (25 cc. containing about 2.1 gm. of enzyme) obtained from 30 cc. of the 10 per cent solutions were used. To this solution at the desired pH was added 1.0 gm. of silica gel. It was then shaken slowly for 30 minutes in the ice box, the silica gel centrifuged, and the supernatant liquid treated with a second portion of the gel. At each successive addition of silica gel, the pH had to be readjusted because this acid adsorbent removed some of the hydroxyl ions. After the desired number of adsorptions the supernatant liquid was precipitated as usual. For testing with glycerol phosphate the supernatant liquid may also be used directly without precipitation. In the case of the aluminum hydroxide adsorbent,<sup>5</sup> 2.64 cc. of a solution containing 0.10 gm. of the hydroxide were added to the centrifuged enzyme solution. This was shaken in the cold for 30 minutes and the hydroxide removed by centrifuging. This process was repeated after the final adsorption and the remaining enzyme was precipitated. Treatment with colloidal ferric hydroxide was accomplished by adding 1.5 cc. of colloidal iron (Merck's dialyzed colloidal iron, 5 per cent  $\text{Fe}_2\text{O}_3$ ) to the enzyme solution. The pH of the solution changed during this addition of iron from 6.5 to 6.0. The coagulated ferric hydroxide was centrifuged off and the enzyme precipitated. The supernatant liquid gave a negative test for iron with hydrogen sulfide. Calcium phosphate<sup>6</sup> was precipitated in the enzyme solution at a pH of  $8.5 \pm 0.2$ . To the enzyme solution were added 10 cc. of 0.25 molal disodium hydrogen phosphate solution and then alternately six portions of 0.5 cc. of 1.25 molal calcium acetate and 0.05 cc. of 5.0 molal sodium hydroxide. In this manner the change in pH was kept within the limits indicated above. After centrifuging the precipitate the supernatant liquid was found to give only a trace of precipitate on further addition of either reagent. The enzyme was recovered as usual. The adsorption by calcium phosphate<sup>6</sup> precipitated at a pH of 6.6 to 7.2 was also tried. In this case 10

cc. of 0.25 molar disodium hydrogen phosphate were added to the enzyme solution and 2.5 cc. of 1.25 molar calcium acetate were added in 0.5 cc. portions. No further precipitation occurred after the last 0.5 cc. addition. The enzyme solution of pH 6.6 changed to 7.2 during this precipitation. The phosphate was removed and the remaining enzyme precipitated with acetone. These experiments are all summarized in Table VI. This shows the adsorption of polynucleotidase that was effected by these reagents, the effect of pH upon the adsorption by silica gel and calcium phosphate, and the effect of the number of adsorptions on the activity adsorbed.

*IX. Elution from Adsorbents*—Attempts to elute the adsorbed enzymes were entirely unsuccessful. In fact, even the suspended adsorbents were inactive both alone and in the presence of active nucleotidase fractions.<sup>12</sup> Thus, for example, the polynucleotidase adsorbed by three or four 1.0 gm. samples of silica gel was that originally contained in approximately 2.0 gm. of once precipitated enzyme. Resuspension of this silica gel in water and using it in a single polynucleotidase assay should have resulted in the hydrolysis of nucleic acid to mononucleotides at a very high rate. Moreover, in the presence of only a small amount of enzyme from which the polynucleotidase had been adsorbed, the nucleic acid should have been completely hydrolyzed to phosphoric acid and nucleosides in a short while. Neither of these results was found in this case nor in the case of aluminum hydroxide adsorptions. Inasmuch as the adsorption of polynucleotidase evidently takes place in the alkaline region with little or none occurring on the acid side (except with ferric hydroxide) elution was attempted by trying to reverse the adsorptions in acid media. This was done with and without the addition of glycerol. The adsorbents were suspended in a small amount of solution and the pH lowered to 4.5 or 5.0 with acetic acid. The supernatant liquid was then removed and adjusted to a pH of 8.6 for the assay. The hydrolysis results were all negative.

<sup>12</sup> It has been shown that the adsorbed enzyme need not necessarily be eluted from the adsorbent in order for it to act on the substrate. Levene, P. A., and Weber, I., *J. Biol. Chem.*, **60**, 707 (1924).



# **FURTHER EVIDENCE FOR THE EXISTENCE OF A THIRD VITAMIN B GROWTH FACTOR FOR THE RAT, PROBABLY VITAMIN B<sub>4</sub>\***

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Reader (1-4) has described a factor (vitamin B<sub>4</sub>) obtained from the decomposed mercuric sulfate precipitate in the Kinnersley and Peters process for the preparation of torulin. This factor supplements vitamins B<sub>1</sub> and B<sub>2</sub> (B and G) when given to rats which are receiving a basal diet lacking in all the B vitamins, and in addition, materials containing the two recognized factors. She has recently worked out a new method of testing for the presence of this vitamin. If animals are raised to adult size on this diet plus the three factors and are then deprived of vitamins B<sub>1</sub> and B<sub>4</sub> until polyneuritis occurs, they can be cured of convulsions and paralysis in a few hours by feeding 2 to 3 pigeon day doses of a potent vitamin B<sub>1</sub> preparation. The weight ceases to fall and is maintained on a level, but the animals show a general muscular weakness, swollen red paws, a tendency to sit in a hunched position, and to walk with a rolling gait. Addition of a material containing vitamin B<sub>4</sub> leads to increased weight and greater activity. In a week the gait is normal. If the sample tested does not contain the vitamin the animals become worse and die in 10 to 12 days.

The present paper describes a series of experiments with results so similar to those of Reader that we are led to believe that we have further evidence for the existence of this vitamin.

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## EXPERIMENTAL

Experiments have been in progress in this laboratory in which attempts were made to determine the effect of heat at varying concentrations of hydrogen ion on vitamin B ( $B_1$ ). The materials used as a source of the vitamin were skim milk powder and protein-free milk, prepared according to a method described elsewhere (5). Albino rats were used, and the technique of Chase and Sherman (6) was followed. All animals came from mothers which had been receiving either a modification of the Steenbock laboratory stock diet, or Diet 13 of Sherman and Campbell (7).

It was noticed that the experimental animals which received these supplements in such amounts as to induce a gain up to about 7 gm. a week uniformly ceased to grow at as good a rate after about the 5th week. Often there was only a gain of 1 to 2 gm. a week after this time. With larger portions there was a tendency toward a more constant growth rate. It seemed probable that some factor was lacking in the basal diet and present to a very small extent in the supplements, and that the animals had a body store of this factor which was depleted in about 5 weeks.

In standardizing the method of testing for vitamin B ( $B_1$ ) Chase and Sherman (6) used whole wheat as a supplement, and in no case was there a flattening of the growth curves such as we obtained. Whole wheat, whole white and whole yellow corn had been tested in this laboratory for their vitamin B ( $B_1$ ) values, and it was found that animals which received the whole wheat grew at a more constant rate than those receiving corn or the milk preparations.

A consideration of these results led us to feed to some rats very small portions (0.2 gm. daily 6 days per week) of whole wheat in addition to the supplement which the animals had received during the 8 week experimental period. This amount of whole wheat contains so little vitamin B ( $B_1$ ) that animals receiving it as the sole supplement to the basal diet become polyneuritic and rarely survive the 8 week experimental period. However, when given in addition to a supplement containing vitamin B ( $B_1$ ), in these experiments, there was a sudden increase in growth. Rats whose weights had become stationary often gained 10 to 12 gm. a week continuously for several weeks. Eight animals which did not receive the whole wheat addition continued to lose weight and

died at about 70 to 80 days from the time they were first placed on the experimental diet.

It seemed possible that the sudden weight increase in these rats was a result of the addition of a third factor. The animals had received a diet which included 15 per cent of autoclaved yeast as a source of vitamin G ( $B_2$ ), while the original supplement presumably provided vitamin B ( $B_1$ ) since animals which grew 3 or more gm. a week and did not show polyneuritis must have received a fair allowance of this factor.

The technique as described by Reader (3) appeared to offer a means of using these animals in a similar way. The method adopted by us was as follows: After the animals had received the whole wheat supplement for 3 to 4 weeks and they weighed about 150 gm., both the original supplement and the whole wheat were removed. In about 2 weeks severe polyneuritis symptoms appeared suddenly, following a very rapid decline in weight. At this point either the original supplement or a preparation of Lloyd's reagent<sup>1</sup> was fed, the portions fed being such as to represent that amount of skim milk powder which had induced a gain of about 5 gm. a week for 5 weeks, with some flattening of the growth curve after this date. It was often necessary to feed the material by hand because of the paralysis and convulsions, and several animals died at this stage. It was possible, however, to cure the condition in about twenty cases, which appeared to justify the conclusion that the material given was potent in vitamin B ( $B_1$ ).

After the animals had been cured of polyneuritis and their weight had remained practically constant for several weeks, 0.2 gm. daily of whole wheat was added. There was again a sudden increase in growth rate. It was possible to use the same animal for several successive tests and the response was similar in each instance, thus showing a correlation with Reader's reported results.

<sup>1</sup> The activated Lloyd's reagent was prepared as follows: To 1 liter of protein-free milk at about pH 4.3, 10 gm. of Lloyd's reagent were added. The material was stirred at intervals and kept at ice box temperature overnight. It was filtered on a Buchner funnel, washed with a little distilled water, and allowed to dry at 50° or lower.

The author wishes to take this opportunity to thank Professor John Uri Lloyd for the generous sample which he sent her.

Three typical cases are shown in Fig. 1. The first animal received a small portion of skim milk powder during the experimental period, and was losing weight. Addition of whole wheat resulted in a rapid gain, as shown. There was a loss of

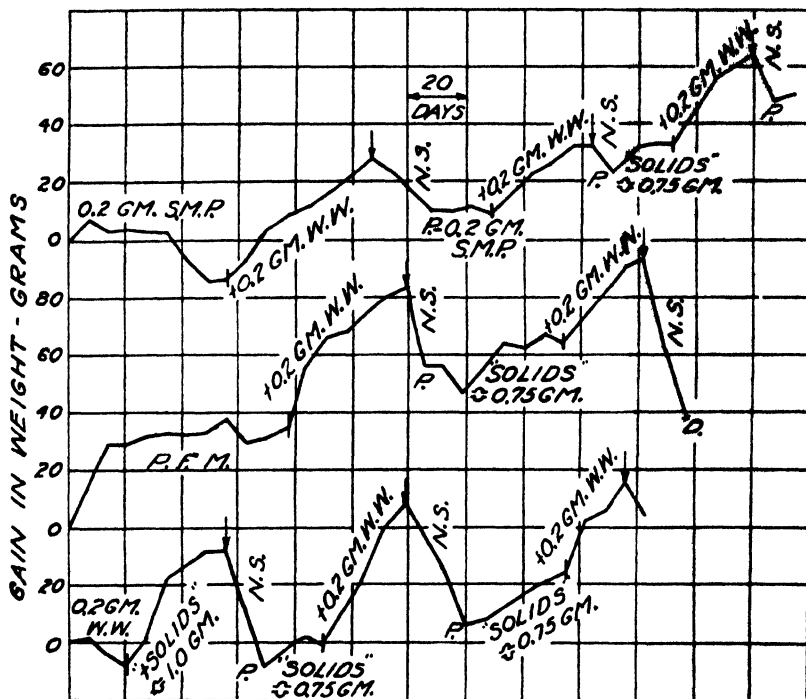


FIG. 1. Growth curves of individual rats which received the vitamin B- (B<sub>1</sub>) deficient diet plus supplements containing this vitamin, with and without the whole wheat addition. At the point indicated by the vertical bar whole wheat was added; at the arrow both supplements were withdrawn; at P. a supplement containing vitamin B (B<sub>1</sub>) was given. S.M.P. represents skim milk powder; W.W., whole wheat; "solids," activated Lloyd's reagent; P.F.M., protein-free milk; N.S., no supplement; P., polyneuritis; D., died.

weight with development of polyneuritis when the supplements were withdrawn. Addition of skim milk powder improved the polyneuritis but growth was not resumed until whole wheat

was given. Both supplements were removed again and when polyneuritis developed it was cured with "solids" in amounts corresponding to 0.75 gm. of skim milk powder. There was a slight gain, followed a third time by rapid growth on addition of whole wheat.

The second animal had received a protein-free milk preparation. There was a rapid gain at first, followed by a long period of weight maintenance. The rapid gain on addition of whole wheat in both periods is seen. The animal died with polyneuritis following the second removal of supplements.

The third animal shown had been treated in a slightly different manner. After polyneuritis had occurred following the use of 0.2 gm. of whole wheat daily as the sole supplement, the "activated solids" were fed. The supplementary action of these two is again seen. This animal was used for two more tests and the response was similar in each case. For lack of space only these three records are presented, but they are representative of about twenty cases. The close similarity between these curves and those of Reader (3) is interesting.

In order to test the question further a new series of animals was started. In this case litter mates received in addition to the basal diet graded portions of either skim milk powder, protein-free milk, or whole wheat, in amounts having approximately the same vitamin B ( $B_1$ ) content, or a combination of half the amounts of whole wheat plus a half portion of skim milk powder or protein-free milk. It was felt that if there were any supplementary action such a combination would lead to better growth than either one alone. That such is actually the case is seen in Fig. 2. With these animals the feeding of 0.2 gm. of whole wheat plus 0.2 gm. of skim milk powder daily led to better gain than 0.4 gm. of either supplement alone, and 0.1 gm. of both was better than 0.2 gm. of either one. In this latter case no animals which received the combined portion died in the 8 week period, although several from the other two groups failed to survive. Similar results were seen with protein-free milk and whole wheat.

Reader reported that some of the symptoms previously considered as typical of vitamin  $B_1$  deficiency in rats were in reality due to a lack of vitamin  $B_4$ . In the present experiments there was not available a concentrate of vitamin B ( $B_1$ ) similar to that

employed by Reader. It was possible for her to feed as high as 10 to 12 pigeon day doses of the concentrate to assure herself that the animals had sufficient vitamin B<sub>1</sub>. Since the skim milk powder from which we prepared our "solids" presumably contained a small amount of the new factor, we felt we could not increase our dosage beyond the amount which would cure severe

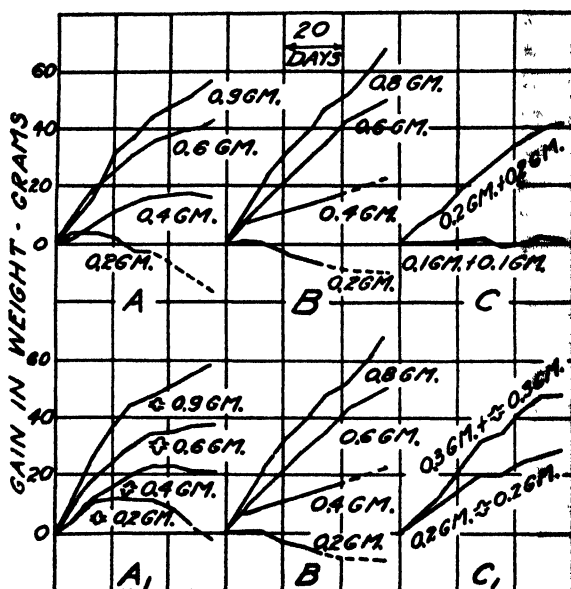


FIG. 2. Average growth curves of rats receiving the vitamin B- (B<sub>1</sub>) deficient diet plus: A, graded portions of skim milk powder; A<sub>1</sub>, graded portions of protein-free milk; B, graded portions of whole wheat; C, half portions of whole wheat plus half portions of skim milk powder; C<sub>1</sub>, half portions of whole wheat plus half portions of protein-free milk. Each curve represents the average of six or more animals. The broken line indicates that one or more animals have died.

polyneuritis. However, it was found that when the animals could eat the supplement and had recovered from the head retraction and convulsions they still walked with a rolling gait and were extremely nervous. The animals did not always show red paws, but they had a tendency to sit in a humped position. All of these symptoms were relieved when the whole wheat was fed. There

was also a marked increase in food consumption showing that the appetite had been stimulated.<sup>2</sup>

#### DISCUSSION

Since it has become established that vitamin B (old nomenclature) is not a single entity, much evidence has accumulated leading to the view that there are probably more than the two originally determined factors. Technique for this work has become more quantitative, so that a consistent variation in growth usually can be taken to mean that some necessary factor is not being provided in adequate amounts.

Coward and coworkers (8, 9) found a substance in milk, lettuce, ox muscle, liver, and wheat embryo which was effective in inducing growth in animals which had ceased to grow although they were receiving a diet adequate in calories, digestibility, and all known vitamins. Heated milk was less effective. Guha (10) has obtained somewhat similar results, using a technique similar to Reader's. He fed animals either a diet lacking in both B vitamins supplemented with vitamin B<sub>1</sub> (B) and B<sub>2</sub> (G) preparations of known potency in adequate amounts, or a diet which included 7 per cent of dry yeast. In both cases he noticed that the growth fell off after about 9 to 10 weeks. He then tested various foods to determine whether continued growth could be obtained. Increased amounts of any of the vitamins already furnished had no effect, nor had orange juice nor any of the essential amino acids. The best sources were fresh yeast, pasteurized fresh milk, egg yolk, spinach, and alfalfa.

Reader's (1-3) work as reported has concerned yeast preparations. Since all of the known B vitamins are present in yeast and also to a greater or lesser degree in various foods, it seems reasonable to assume that her factor may be found in natural foods. Whether it is analogous with the factor or factors described by Coward *et al.* or by Guha or ourselves, further work alone will show. Guha has found his factor to be readily destroyed by heat. Coward, Key, and Morgan, however, found it possible to extract their factor from wheat embryo with boiling 90 per cent alcohol or with ether. Reader found that vitamin B<sub>4</sub> was most readily extracted by 50 per cent alcohol, but it can be extracted with ether.

<sup>2</sup> Careful food records were kept for all experimental animals. For lack of space tables presenting such data are not included.

Experiments are in progress in this laboratory which represent an attempt to extract the factor from whole wheat by these various solvents.

We have attempted to correlate these results with those reported by Hunt and coworkers (11-13). However, the methods of study have been so different that until more is known of the various factors it would be difficult to determine whether we are dealing with the same vitamin.

It is recognized that the work as here reported is far from complete, but it was felt that this preliminary note might be of interest and help. In a later communication it will be shown that the factor (or factors) present in whole wheat might well be included in the basal diet when determinations of the vitamin B ( $B_1$ ) content of a substance are to be made. Natural foods may or may not contain an adequate supply to supplement the animal's bodily store and the basal diet, but when tests are to be made on more or less highly purified materials, it would seem advisable to supply the new factor.

#### SUMMARY

With the technique described by Reader, evidence is furnished for the existence of a factor (or factors) in a natural food material, supplementing vitamins B ( $B_1$ ) and G ( $B_2$ ), the results being such as to lead to the conclusion that the factor probably is vitamin  $B_1$ .

A discussion is included in which these results are also compared with those reported by Coward and coworkers, Guha, and Hunt.

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## THE VALIDITY OF DETERMINATIONS OF THE COLLOID OSMOTIC PRESSURE OF SERUM

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Measurement of the colloid osmotic pressure of the blood, or argument depending thereon, has been involved in much recent work on the origin of tissue fluid from the blood through capillary walls and in work on changes taking place in the blood due to various causes. It may be sufficient to mention Krogh (1929), Landis (1925-26, 1927, 1930, *a*, 1930, *b*), Loewen, Field, and Drinker (1931), Meyer (1931, *a*) and Clark and Holling (1931). The colloid osmotic pressure of the blood has been found of especial significance in edema formation (Govaerts, 1924, 1927; Schade and Claussen, 1924; Schade *et al.*, 1926; Kylin and von Pein, 1931; Meyer, 1931, *b*; Iversen and Nakazawa, 1927; Meulengracht, Iversen, and Nakazawa, 1928; and others). It, therefore, becomes increasingly necessary to obtain valid and workable methods for measuring colloid osmotic pressure. Two relatively simple methods described by Krogh and Nakazawa (1927) have been much used. They, like other methods for this purpose, involve the use of artificial membranes and the objection has been raised that the colloid osmotic pressure obtained by such laboratory methods is not the same as the colloid osmotic pressure we would like to measure, that effective in the capillaries, because the membrane of the capillary wall and the artificial membranes used in the laboratory are very unlike, and especially it is claimed they may be fatally unlike in that the electrical properties of the membranes may invalidate the readings secured by their use.

While it is obviously true that the actual osmotic conditions

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cannot be reproduced in the laboratory and while further it is true that at present accurate knowledge is incomplete concerning either the general permeability of capillary walls to protein or the variations in permeability which may occur at different times and places (Conklin, 1930; Field and Drinker, 1931; Drinker and Field, 1931), all investigators are agreed on the importance of the pull of the plasma colloids in the control of the volume of tissue fluid in mammals. Thus though the actual operative colloid osmotic pressure cannot be known at present, it is of the utmost importance to ascertain comparative pressures in different bloods and in the blood of a single subject at different times. It is essential that the methods used be capable of yielding reproducible readings under identical conditions and that the permeability of the membranes used be known.

Given apparatus technically suitable, the burden of the determination must always rest on the membrane employed. It was to determine the reliability of different membranes that the work described below was undertaken. While the capillary wall may not be protein-tight, the most feasible and generally accepted standard for membranes seems one which permits the free passage of crystalloids but prevents the movement of colloids. If several such protein-tight membranes could be found, differing widely in their nature, and if a series of determinations made on serum under identical conditions, save for the use of different membranes, should give practically the same figures for the colloid osmotic pressure of the sample in question, there would be at least a strong indication that this is the true colloid osmotic pressure of the serum used and also that these membranes do not distort the result by their varying electrical properties.

The membranes which were tried are listed in Table I, with statements as to their protein tightness and suitability for the type of osmometer used. The list includes membranes used by various workers who have measured colloid osmotic pressure for different physiological purposes. The tests were made according to the second method of Krogh and Nakazawa (1927), which employs flat membranes held tightly in place, with the serum above connected with a capillary upon which the necessary counterbalancing pressure exerted by a column of water can be measured. The outer liquid is borne by filter paper below the membrane. It

is small in amount and thus rapidly equilibrated. Certain modifications of the apparatus as originally described and additional precautions are given in notes at the end of this paper for the benefit of those who may wish to use the method. In these experiments horse serum was used as the inner liquid and unless otherwise stated 0.9 per cent sodium chloride as the outer liquid. The tests for protein tightness of the membranes were made in two ways: (a) by filtering either egg albumin solution or horse serum through the membrane in question in a Thiessen filtration apparatus (from Membranfilter Gesellschaft, m.b.H., Göttingen) under a pressure of 2 to 2½ atmospheres and testing the filtrate by Spiegler's reagent;<sup>1</sup> (b) by testing, after an osmometer had been in use for 24 hours or more, the filter paper carrying the outer liquid which had been below the filtration membrane. The papers were soaked for this test in a very little 0.9 per cent sodium chloride and this was then tested by Spiegler's reagent. In this second test the osmometer was taken apart with the utmost care so that the filter papers might be lifted out without the movement of the membrane above. If the membrane were dislodged by even the slightest amount there was practically always contamination by the serum above the membrane and hence invalidation of the test.

From Table I it is seen that three membranes gave identical results: (a) the *Ultrafeinfilter* of Zsigmondy, especially the 110 and 120 minute grades, (b) cellophane No. 600,<sup>2</sup> (c) so called fish bladder, presumably the prepared cecum of the rabbit. It is possible that the results with the heavy parchment shell warrant its inclusion in the list since the readings obtained from its use differed by only 15 mm. from the other good membranes, but its texture is not suitable.

Data from a comparative trial of the three good membranes are given in Table II. The inner liquid in this case was horse serum. There were two outer liquids, one the usual 0.9 per cent sodium chloride, the other an ultrafiltrate from horse serum obtained by

<sup>1</sup> The formula for Spiegler's reagent is 4 gm. of mercuric bichloride, 2 gm. of tartaric acid, 10 gm. of glycerol, and 100 gm. of distilled water.

<sup>2</sup> In this work *Cellophan* No. 600, obtained from Kalle and Company, A. G., Biebrich, Germany, which is identical to the du Pont cellophane bearing the same preparation number, was used.

TABLE I  
*Reliability of Various Membranes for Colloid Osmotic Studies*

Membrane No.	Membrane used	Protein tightness	Comments and notes
1	<i>Ultrafeinfilter</i> (Zsigmondy) 100 min. grade  110    "    "  120    "    " 150    "    "  650    "    "	Rarely slightly permeable Protein-tight  " "  "	From Membranfilter Gesellschaft, m.b.H., Göttingen  1 sheet tested at factory by Congo red and protein was best used Several sheets used. Good 2 sheets used. Rather slow in reaching equilibrium Very high readings even after 24 hrs. Either very slow or impermeable to substances other than colloids. Not suitable
2	Cellophane   No. 400  "    600	Occasionally slightly permeable  Protein-tight	Product from Kalle and Co., A. G., Biebrich, Germany. Same product obtainable in U. S. with same numbers. Made by du Pont Cellophane Co., Inc., New York Occasional permeability perhaps due to delicacy of membrane and hence to injury, or to irregularity in product Good. Must be cut wet, since wet and dry measurements differ, especially in one diameter
3	Ultrafilter  4.5 per cent 6.0    "    "  7.5    "    "	Not protein-tight Protein-tight  "	Schleicher and Schüll, according to Bechhold. Impregnated <i>in vacuo</i> with acetic acid collodion  Not suitable because too thick. Membrane holds too much liquid in itself which may be squeezed into upper chamber of osmometer at time of closing See note above

TABLE I—*Concluded*

Membrane No.	Membrane used	Protein tightness	Comments and notes
4	Parchment paper	Easily permeable	<i>Dialysator-Filter</i> , No. 521, Schleicher and Schüll. No readings possible
5	Parchment shell	Protein-tight	<i>Diffusions-Hülsen</i> , No. 579, Schleicher and Schüll. Large size, 35 × 100 mm. Circles cut from this. Not suitable because too thick. See Membrane 3; readings differed very little from those of Membranes 1 and 2, 15 mm. higher at usual times of reading
6	Mesentery of dog, fresh	Easily permeable	No holes visible on careful examination with hand lens either before or after use. No readings possible
7	Membrane from hen's egg	Easily permeable	Small egg, delicate membrane. No holes visible by hand lens in pieces used though seen in other pieces. No readings possible
8	Pig bladder, dried	Not protein-tight, less permeable than Membranes 6 and 7	As obtained through H. Struers, Copenhagen. Slaughter-house product. Pieces soaked until protein-free. Clumsy to use, too thick. See Membrane 3
9	Prepared rabbit cecum (?). So called fish bladder	Protein-tight	As obtained through H. Struers, Copenhagen. Very thin and flexible. Pieces washed until protein-free. Examined carefully for minute holes, occasionally found. Good results

the use of the Thiessen apparatus with No. 600 cellophane. A preliminary reading was made on each tube about 3 hours after

it was set up. Two or three readings were made after equilibrium was established within the period 9 to 23 hours after the experiment was set up. These readings on a single osmometer varied from each other by from 1 to 8 mm. of water pressure, with an average difference of 3.9 mm. Table II gives the averages of the readings from individual osmometers and the averages for the tubes in each group containing the same liquids.

Thus the same result, within the limits of error of the method (estimated by Professor Krogh at not more than 10 mm.), was

TABLE II

*Data from Comparative Trial of Three Good Membranes*

All figures are the averages of two or three readings on single osmometers unless otherwise stated.

Membrane used	H <sub>2</sub> O pressure with outer liquid ultrafiltrate	H <sub>2</sub> O pressure with outer liquid 0.9 per cent sodium chloride
	mm.	mm.
Cellophane No. 600	257 247 253	261 250
Average.....	252 (for 3 osmometers)	255.5 (for 2 osmometers)
Ultrafeinfilter, 120 min. grade	256 248	252 249
Average.....	252 (for 2 osmometers)	250.5 (for 2 osmometers)
Rabbit cecum (?) "fish bladder"	244 251 250	244 247 249
Average.....	248 (for 3 osmometers)	247 (for 3 osmometers)

obtained from *Ultrafeinfilter*, a membrane with a collodion basis; from one based on pure cellulose hydrate (statement by manufacturers of cellophane); and from the very different animal membrane. Not only was the same result obtained in these cases but the time at which equilibrium was reached was practically the same, though steady readings lasted somewhat less long with the third membrane. This essential identity of behavior indicates that under the conditions of the test we have in these three cases membranes which will give with 0.9 per cent sodium chloride or an

ultrafiltrate as an outer liquid a ready adjustment of the blood crystalloids and which very probably give the true colloid osmotic pressure of serum. They thus meet the qualifications for good membranes which were established at the start, impermeability to colloids and ready permeability to the other constituents of serum. Further, they can be assumed to be free from systematic errors due to their electrical properties. For practical purposes *Ultrafeinfilter*, 110 or 120 minute grades, and cellophane No. 600 are well adapted.

A few tests were made on the effect of using oxalate, citrate, and fluoride to prevent coagulation. If not more of the oxalate was used than was really required for the purpose, there seemed little or no effect on the osmometer readings, but if the amount of oxalate was excessive, very high readings might be obtained, perhaps because of failure of the membrane to permit equilibrium in the time usually sufficient, perhaps because of some change in the electrical condition of the membrane. The use of citrate and fluoride seemed to be accompanied by irregular results. The danger of an alteration in the electrical condition of the membrane would seem particularly probable with the polyvalent citrate ion. It therefore was judged safest to use no anticoagulant but to centrifuge the clotted blood. Certainly the use of oxalate is attended with danger unless it be employed with the utmost precision. As in the work of Krogh and Nakazawa, excess sodium chloride and glucose were found to have no effect on the readings. The use of 0.9 per cent sodium chloride as the outer liquid rather than the calcium-containing Ringer's solution avoided the danger mentioned by Meyer (1931, b) of the precipitation of oxalate on the membrane by the calcium of the outer liquid and hence of an altered permeability.

#### SUMMARY

In order to test the reliability of colloid osmotic pressure determinations on serum or plasma, many different membranes were tried in the Krogh and Nakazawa osmometer. With horse serum as an inner liquid and either its ultrafiltrate or 0.9 per cent sodium chloride as outer liquid, identical results were obtained for the colloid osmotic pressure with the protein-tight membranes, *Ultrafeinfilter* (Zsigmondy) of 110 and 120 minutes, cellophane No. 600,

and the prepared cecum of the rabbit (?) known as fish bladder. This identity of result with such diverse membranes indicates that, since the proteins are known to be held back, the membranes must make such an adjustment of the other serum constituents that the true colloid osmotic pressure is satisfactorily determined by this relatively simple method. The identity of result, further, makes very improbable any distortion of the readings by electrical properties of the membranes.

It is a pleasure to express thanks to Professor August Krogh who suggested this study and who gave most helpful counsel as it proceeded.

#### *Notes on Use of Apparatus*

The following changes were made in the apparatus as first described by Krogh and Nakazawa<sup>3</sup> (see Fig. 1).

1. An additional washer was added below the silver plate to insure the integrity of the air chamber in the hard rubber cone.

2. The lower cylindrical portion of the osmometer chamber was deepened to 6 mm. in order to accommodate more adequately the parts which must go into it (upper washer, filtration membrane, filter papers, silver plate, lower washer) and also to have left a shallow depression into which the end of the cone can fit. This avoids slipping of the parts when the apparatus is screwed together and thus prevents leaks.

3. An arrangement for setting up a set of six osmometers was employed (adapted from that used by Meyer<sup>4</sup> of Altona). See Fig. 2. The rubber tube, *A*, from the pressure system leads to a brass tube, *B*, 24 cm. long and 13 mm. in diameter. From this branch six short tubes, *C*, 25 mm. long and 4 mm. in diameter. All measurements are outside dimensions. The six tubes are included in a space of 20 cm. which brings the osmometers as close together as they can hang conveniently. From each side tube an osmometer is hung by two sections of rubber tubing with a section of glass tubing between them, all of small diameter and short, *D*. The two sections of rubber make possible the clamping off and removal of any osmometer without influence on the pressure in

<sup>3</sup> Now obtainable from Membranfilter Gesellschaft, m.b.H., Göttingen, Germany.

<sup>4</sup> Personal communication to Dr. P. Rehberg.

the system. Small but efficient metal clips and clamps are used in closing off the osmometers. The osmometers hang into a glass jar, *E*, of convenient dimensions and their lower portions dip into 0.7 per cent sodium chloride. The glass jar is supported in a



FIG. 1. Diagram of osmometer. Chamber *D* for the liquid to be tested is in a hard rubber section, *C*. In setting up the osmometer, section *C* is first inverted and the upper rubber washer *J*, filtration membrane (shown by upper line), filter paper carrying the outer liquid (shown by second line), perforated silver disc *G*, lower rubber washer *J*, and hard rubber conical section *I* are added in order and firmly clamped in position by the hard rubber screw *H*. The chamber *D* is then carefully filled entirely full with the liquid to be tested. The capillary *A* with rubber washer *F* at the correct level is pushed through the tubular portion of section *C* to the position shown, the level of the column is adjusted to a convenient height, and the metal nut *E* securely tightened. Rubber tube *B* at the top of the capillary is added and closed by a small metal clip while the apparatus is attached to the pressure system, as shown in Fig. 2.



wooden frame on the base of which rests the stand carrying the brass tube mentioned above. The wooden frame also carries a shelf for the horizontal microscope arranged at a convenient height for reading the tubes and provided with both horizontal and vertical rack and pinion adjustments. The eyepiece contains a micrometer for accurate observation of the liquid column.

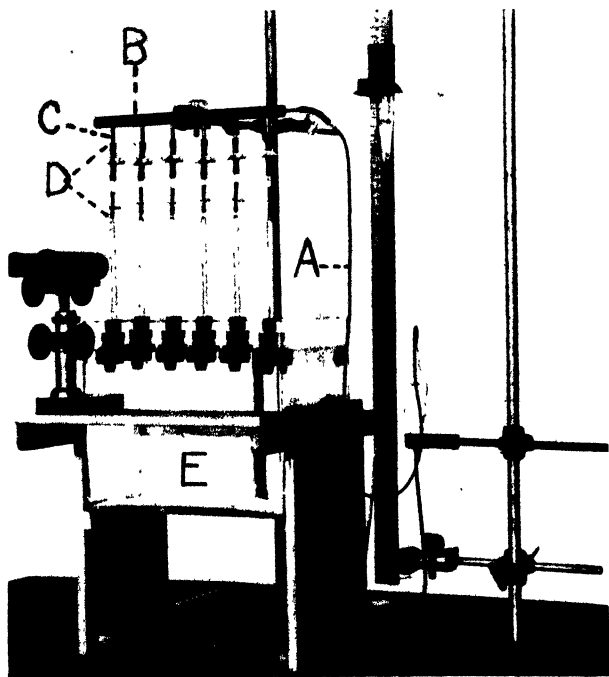


FIG. 2. Set-up of the apparatus for which the description is given in the text.

A few changes in procedure, also, were made for greater safety or convenience.

1. The filter papers which carry the outer liquid were cut by a cork borer one size smaller than that used for the filtration membrane. This lessened the danger of pressing the outer liquid over the edge of the filtration membrane and of its going by capillarity into the upper chamber at the time the system was clamped together.

2. Two filter papers of ordinary thickness seemed to hold a better amount of outer liquid than one. One frequently was found too dry at the end of an experiment, a condition accompanied by irregular results and by very slow setting of the levels during readings.

3. There is always danger of the formation of bubbles in the dry and also invisible interior of the osmometer chamber at the time of filling with serum. These may stick persistently to the hard rubber surface, or, worse, to the membrane, or they may rise later into the capillary. They were avoided by using a slender filling pipette with a smoothly rounded tip and by moving the tip around as filling went on. Bubbles then rose at once to the top and could be easily removed before the capillary was inserted.

4. When the osmometers were set up pressure was put on gradually. This avoided any sudden fall of level in the capillary tube. Such a sudden fall might be accompanied by a drainage so much slower as to lead to the later formation of small bubbles at the top of the column in the tube.

5. Reading the tubes early, before equilibrium was fully attained, seemed to help to a more speedy and regular adjustment. A pressure fairly near the final value was thus put on the tubes about 3 hours after they were set up.

6. The best time for readings was found to be between 6 and 20 hours after the osmometers were set up. Three readings were usually made in this interval, spaced according to convenience. There is time enough for more readings if a tube comes into equilibrium more slowly than usual. Some tubes lasted with steady readings for more than 30 hours, but this was not to be depended upon.

7. A sterile technique was not used. There was no indication of deterioration before the time mentioned above, at the ordinary laboratory temperature and with well washed utensils and hands. All parts of the apparatus were washed thoroughly with tap water, rinsed repeatedly in distilled water, and then in alcohol. They were carefully drained and then dried for 2 to 4 hours in an oven at 60–70°. Capillary tubes were cleaned with dichromate solution frequently, but not after each using. Parts of the apparatus, which were to come into contact with inner or outer liquids, were touched only with utensils cleaned as was the apparatus. Blood

tubes were cleaned in the same way. Trypaflavine was not found necessary.

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# THE TITRATION CONSTANTS OF $\alpha,\beta$ -DIAMINOPROPIONIC ACID AND THEIR RELATION TO THE CONSTANTS OF VARIOUS ISOMERS

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## INTRODUCTION

Beginning with the early work of Ostwald (11) and of Bredig (3), numerous attempts have been made to find some relation between the ionization constants of organic acids and bases and their chemical constitution. Most of the work up to 1913 has been adequately summarized by Lehfel'dt (8), the general consensus of opinion admitting that some more or less regular effect exists between the nature and position of the ionizing group in the organic molecule and the magnitude of its ionization. Such a generalization is portrayed in the table compiled by Wegscheider (18). More recently, Adams (1), Bjerrum (2), Simms (17), and MacInnes (10), to mention only a few, have made important theoretical contributions to the subject, for which the original papers should be consulted. The problem of the influence of substituted groups upon the ionization of amino acids has been extensively analyzed by Cohn (4) on the basis of the present existing data. In general, and in particular among the substituted derivatives of aliphatic acids and bases with which the present paper is concerned, it may be stated that the ionizing capacity falls off with the distance between the groups and is moreover a function of the nature of such groups.

In a consideration of the effect of an  $\alpha$ ,  $\beta$ , or  $\gamma$  substitution in the carbon chain of aliphatic acids, MacInnes (10) demonstrated a remarkable linear relation between the logarithm of the dissocia-

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tion constant and the reciprocal of the distance,  $d$ , between the carboxyl and the substituted group. This distance was arbitrarily designated as 1 for an  $\alpha$  substitution, 2 for a  $\beta$ , 3 for  $\gamma$ , etc. This straight line relationship is represented by the formula

$$\text{Log } K = C + S \frac{1}{d} \quad (1)$$

where  $K$  is the ionization constant of the substituted acid and  $C$  and  $S$  are constants. MacInnes further pointed out the analogy of such a relationship with that of the case of two similarly charged spheres which when brought near to each other will gain in mutual potential energy by an amount inversely proportional to the distance between them. Schmidt, Appleman, and Kirk (13) later pointed out that the same linear relation held in the case of successively  $\alpha$ -,  $\beta$ -,  $\gamma$ -, and  $\delta$ -substituted amino acids. It is apparent, therefore, that the relation between the dissociation of substituted groups in these isomeric aliphatic carbon chains and the relative position of the groups to each other may be expressed in terms of a simple linear formula. This effect should apparently hold if equality of the carbon bonds is assumed and unless bending of the chain occurs—the latter event being unobserved with the substances studied which did not proceed further than an  $\epsilon$  substitution.

The present study forms part of a series of investigations (5, 6) on the influence of the position of those groups found in the protein molecule upon their dissociation. In the proteins it is probable from recent x-ray studies that the constituent amino acids are not arranged in random distribution but are most likely coupled in definite patterns.<sup>1</sup> The dissociation, therefore, of the free groups of the constituent amino acids of the protein must be mutually influenced in a manner which should be conveniently followed by a study of simpler synthetic models. It has been pointed out that the groups of certain amino acids and peptides containing a large number of basic amino acids dissociate at more acid reactions than can be accounted for on the basis of the dissociation constants of the free amino acids (4, 5). The effect must lie both in the mutual influence of the basic groups upon each other and upon the distance between them. The present paper is concerned with

<sup>1</sup> For discussion see Rimington (12).

the effects of introducing an amino group into  $\alpha$ -amino acids in successively  $\beta$ ,  $\delta$ , and  $\epsilon$  positions in the carbon chain, and the relation between the dissociation values of these compounds and those of their respective isomers among the mono-substituted amino acids, the methylene diamines, and the dicarboxylic acids. These effects have been compared with the aid of the MacInnes equation.

Inasmuch as there are no dissociation values of  $\alpha$ ,  $\beta$ -diaminopropionic acid listed in the literature, it was necessary to prepare and study the ionization of this compound. The values for  $\alpha$ ,  $\delta$ -diaminovalerianic acid (ornithine) and of  $\alpha$ ,  $\epsilon$ -diaminocaproic acid (lysine) were taken respectively from the results of Schmidt, Kirk, and Schmidt (15) and Schmidt, Kirk, and Appleman (14). Dissociation values of the methylene diamines were taken from the work of Bredig (3) and of the dicarboxylic acids from Simms (17). All values are given at 25°.

#### EXPERIMENTAL

The monohydrochloride of  $\alpha$ ,  $\beta$ -diaminopropionic acid was prepared according to the method of Klebs (7). It was crystallized twice from alcohol-water mixtures and gave on Cl analysis, 25.02 per cent, theory 25.22 per cent; melting point 226°, uncorrected.

Exactly 0.0703 gm. of the substance was dissolved in 5 cc. of 0.1 M HCl and titrated with N NaOH. Details of the procedure, potentiometric assembly and calibration, together with the method of calculation are given in a previous paper by the writer (5). The activity coefficients of  $H^+$  and  $OH^-$  for the calculation of acid and base bound were experimentally determined in HCl and NaOH solutions containing the same concentration of sodium chloride as was present in the solution of the amino acid.

In Fig. 1 the ordinates represent the acid and base bound by the ampholyte, the abscissæ the pH. The experimental points are indicated and the curves calculated from the titration constants chosen. The constants were calculated according to the *Zwitter Ion* concept, whereby acid groups dissociate at acid reactions and basic groups hydrolyze at alkaline reactions. The values of Schmidt and his coworkers for the other diamino acids and for the substituted monoamino acids were recalculated on this basis by Cohn (4) and the values for the dissociation constants in this communication are identical with those in his compilation. The value

for  $pK_w$  of water at  $25^\circ$  was taken as 13.998. Following the convention of Levene and Simms (9) the constants were numbered  $pG_1'$ ,  $pG_2'$ , etc., the order following from the most acid to the most alkaline value.

The dissociation of each free group is represented by  $\alpha$  in the mass action expression for the behavior of a monovalent acid or base,

$$G' = H \frac{\alpha}{1 - \alpha} \text{ or } pG' = pH + \log \frac{1 - \alpha}{\alpha}$$

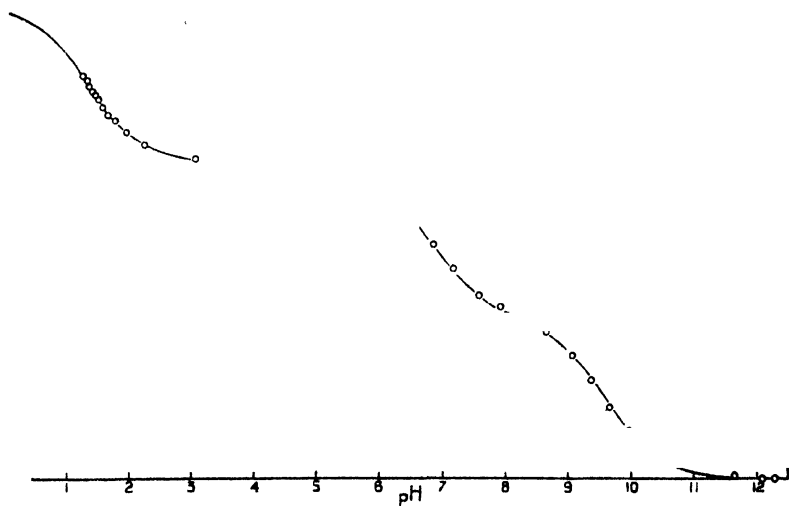


FIG. 1

where  $G'$  is the titration constant related to the classical dissociation constant  $K'$  by an expression first clearly presented by Simms (16). For the sake of consistency all dissociation values cited in the present paper are given as  $pG'$ , this procedure being unobjectionable inasmuch as the groups of the substances considered dissociate at reactions so sufficiently separated in value as to make the difference between  $pG'$  and  $pK'$  negligible.

The apparent titration constant  $pG'$  of each isolated group may be then calculated from the pH at which that group is half neutralized. When, however, two or more groups dissociate simul-

taneously within the same pH range, the  $pG'$  value of these groups must be so chosen that at each pH value that the experiments re-

TABLE I  
 $\alpha, \beta$ -Diaminopropionic Acid (0.1 M)

Concentration of HCl or NaOH	E.M.F.	pH	p $\gamma$	$X$ (experimental)	$X$ (calculated)			
					$pG_1' = 1.33$ $\alpha_1$	$pG_2' = 6.80$ $\alpha_2$	$pG_3' = 9.80$ $\alpha_3$	Total $\Sigma\alpha = x$
<i>N</i>								
0.218	0.4105	1.26	0.07	0.45	0.459			0.46
0.207	0.4144	1.33		0.48	0.500			0.50
0.200	0.4156	1.35		0.52	0.512			0.51
0.190	0.4189	1.40		0.55	0.541			0.54
0.182	0.4216	1.45		0.57	0.569			0.57
0.174	0.4244	1.50		0.60	0.597			0.60
0.163	0.4292	1.58		0.65	0.641			0.64
0.151	0.4342	1.66		0.70	0.682			0.68
0.141	0.4410	1.78		0.74	0.739			0.74
0.125	0.4519	1.96		0.81	0.811			0.81
0.110	0.4688	2.25		0.89	0.893			0.89
0.095	0.5177	3.07		0.98	0.987			0.99
0.081	0.6777	5.83		1.10		0.097		1.10
0.077	0.6905	6.00		1.14		0.137		1.14
0.054	0.7244	6.57		1.39		0.371		1.37
0.041	0.7409	6.85		1.53		0.529		1.53
0.028	0.7594	7.16		1.68		0.696		1.70
0.014	0.7842	7.58		1.85		0.858		1.86
0.007	0.8042	7.92		1.92		0.925	0.020	1.95
0.007	0.8469	8.65		2.08		0.986	0.101	2.09
0.019	0.8722	9.07	0.15	2.23			0.228	2.23
0.031	0.8900	9.37		2.38			0.371	2.37
0.043	0.9070	9.66		2.54			0.535	2.54
0.054	0.9259	9.98		2.70			0.706	2.71
0.066	0.9570	10.51		2.88			0.891	2.89
0.078	1.0238	11.64		2.98			0.991	2.99
0.089	1.0496	12.07		3.00			1.000	3.00
0.099	1.0610	12.27		3.00			1.000	3.00

veal, the sum of the dissociation stages,  $\Sigma\alpha$ , of all such groups will yield the mols of acid or base combined,  $x$ , by the ampholyte:  $\Sigma\alpha = x$  (Table I).



## DISCUSSION

The relative effect of an amino group substitution in  $\alpha$ -amino acids and its position in the molecule is clearly shown in Table II.

The introduction of the second basic group causes the greatest effect upon the acidity of the molecule when such substitution takes place in the  $\beta$  position and decreases in effect with successive  $\delta$  and  $\epsilon$  substitution. In  $\alpha$ ,  $\beta$ -diaminopropionic acid where the basic groups are joined to neighboring carbon atoms, the highest acid values are found; *i.e.*, the carboxyl group dissociates at the highly acid reaction of 1.33 and the  $\alpha$ -amino group at the slightly acid value of 6.80. The order of magnitude of these constants is exceptional for the amino acids although certain of the latter compounds

TABLE II  
*Titration Constants at 25°*

	Carboxyl	Amino		$pG_2' - pG_1'$	Isoelectric point
$\alpha, \beta$ -Diaminopropionic acid	$\frac{pG_1'}{1.33}$	$\frac{pG_2'}{6.80}$	$\frac{pG_3'}{9.60}$	2.80	$\frac{pI}{8.20}$
$\alpha, \delta$ -Diaminovalerianic acid	$\frac{pG_1'}{1.94}$	$\frac{pG_2'}{8.65}$	$\frac{pG_3'}{10.76}$	2.11	$\frac{pI}{9.70}$
$\alpha, \epsilon$ -Diaminocaproic acid	$\frac{pG_1'}{2.17}$	$\frac{pG_2'}{8.94}$	$\frac{pG_3'}{10.53}$	1.59	$\frac{pI}{9.74}$

possessing extra electronegative groups such as histidine and phenylalanine have yielded fairly acid values for the dissociation of the carboxyl group. As would be expected from the proximity of highly charged and similar groups, the difference in the dissociation values of the amino groups in the diamino acids is greatest when they are nearest to each other. However, as the terminal amino group is substituted successively on  $\delta$ - and  $\epsilon$ -carbon atoms, the basicity of the molecule increases, the isoelectric points shift to a more alkaline range, and the difference in dissociation values of the amino groups diminishes. This phenomenon was pointed out by Bredig ((3) p. 326) who said, "dass die basischen Eigenschaften . . . mit zunehmender Entfernung der beiden Amidogruppen resp. der beiden elektrischen Ladungen voneinander zunehmen."

The influence upon the carboxyl in these diamino acids is due to the extra basic group, inasmuch as the  $\alpha$ -amino group preserves a constant position in the molecule. If we follow the procedure of MacInnes and plot the  $\text{pG}'$  values of the carboxyl groups as inverse functions of single whole numbers, arbitrarily designated as the distance between carboxyl and terminal amino groups, then a straight line may be drawn between the points (Fig. 2, Curve I). The distance  $d$  is assumed, following MacInnes, to have the value of 2 for the  $\beta$  position of the terminal amino group, 4 for the  $\delta$ , and 5 for the  $\epsilon$ , these numbers referring to the number of carbon atoms separating the two groups. The straight line drawn varies in no case more than 0.05  $\text{pG}'$  from the points, a result well within the experimental error. On the other hand we have but three points in these series, which so fall that although they do not prove the straight line relation they can be described in terms of it. The equation for this line yields

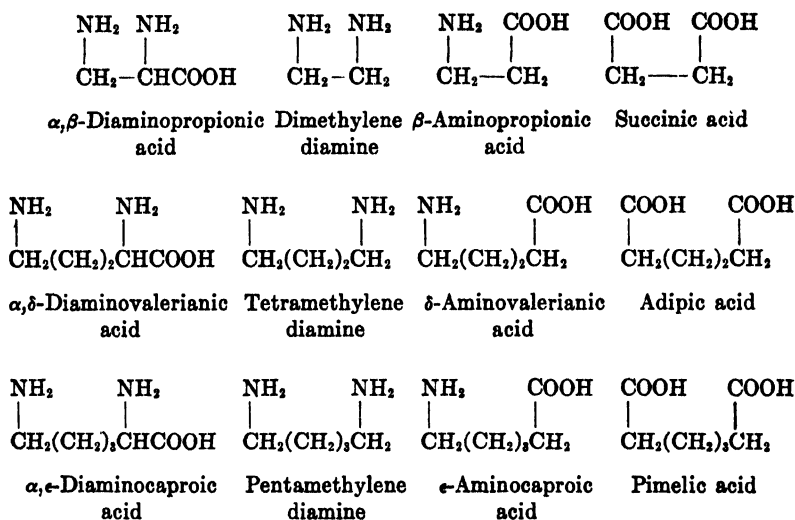
$$\text{pG}' = 2.64 - 2.50 \frac{1}{d}$$

where the constant 2.64 may be assumed to be the value for the carboxyl group when the terminal amino group is removed to an infinite distance along the carbon chain. In such a case the carboxyl should be influenced only by the  $\alpha$ -amino group and should yield the ordinary value of an  $\alpha$ -amino acid in the neighborhood of 2.3. The discrepancy between the value generally found for the carboxyl group of an  $\alpha$ -amino acid and this extrapolated value cannot at present be satisfactorily explained. That it probably is not primarily due to a bending of the carbon chain, bringing the terminal basic group near the carboxyl, is demonstrated below in the behavior of the mono-substituted amino acids.

MacInnes pointed out (10) that the term  $S \frac{1}{d}$  in Equation 1 was "a measure of the potential energy involved in bringing the two negative groups near to each other." The slope  $S$  must therefore be a measure of the influence of one group upon the ionization of another, and should be identical for the same influencing group. So, for example, MacInnes found different values of  $S$  for chloro-substituted and for hydroxy-substituted aliphatic

acids, whereas  $S$  was identical for the chloro-substituted aliphatic and aromatic acids and practically the same for the bromo- and iodo-substituted acids. It is further indicated below that this effect is independent of the nature of the group acted upon, i.e. the ionizing group.

In order further to test this hypothesis, the dissociation values of the diamino acids, of the mono-substituted amino acids, the methylene diamines, and the dicarboxylic acids were compared with the aid of the MacInnes equation. The relation of the isomeric substances to each other is given by the following formulæ.



In this series, the effect of the substitution of an amino group upon the dissociation of the carboxyl group can be studied in the diamino and monoamino acids and likewise the effect of one amino group upon another in the dimethylene diamines. Moreover, the influence of a carboxyl group substitution upon the dissociation, respectively of amino and carboxyl groups, can be observed in the monoamino acids and in the dicarboxylic acids. The dissociation values of these compounds are plotted as inverse functions of the distance in Fig. 2. In the latter, the graphical representation of the monoamino acids differs from that originally presented by Schmidt, Appleman, and Kirk (13) in that in the

present paper dissociation values calculated on the *Zwitter Ion* basis ((4) p. 842) are employed.

The straight lines, Curves I, II, and III, are drawn parallel to each other as are Curves IV and V.<sup>2</sup> They satisfactorily describe

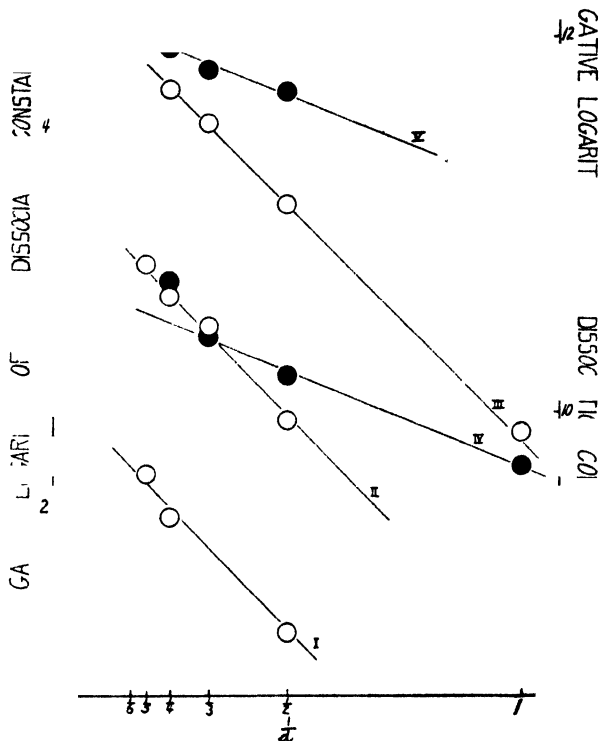


FIG. 2

the experimental material. The first three curves illustrate the influence of an identical group; namely, that of the amino group

<sup>2</sup> The values plotted of the dicarboxylic acids are those of the primary carboxyl group and begin with succinic acid. It is evident that the value for malonic acid does not fit in the plot which may be due to the fact that both carboxyl groups are attached to the same carbon atom. It is likewise noteworthy that the values for the secondary carboxyl group of these acids beginning with succinic acid possess practically a constant value.

upon the ionization of either a carboxyl or of another amino group. Again, the second two curves illustrate the influence of the carboxyl group upon the dissociation respectively of the amino and of the carboxyl group. The slopes for identical substituents appear to be identical and are independent of the nature of the ionizing group.

In the case of the relation between the acid dissociation of the diamino acids and of the monoamino acids, this parallelism is clear. The difference in the  $pG'$  values for the carboxyl group dissociation in the diamino acids and in the monoamino acids at identical positions of the terminal amino group should be an indication of the effect of the introduction of an  $\alpha$ -amino group into the aliphatic acid molecule. That this effect is constant due to the fixed position of the latter group in the molecule is not surprising.

The equations corresponding to the curves of Fig. 2 are:

$$\text{Curve I, } pG' = 2.64 - 2.50 \frac{1}{d} \quad (2)$$

$$\text{" II, } pG' = 11.26 - 2.50 \frac{1}{d} \quad (3)$$

$$\text{" III, } pG' = 4.83 - 2.50 \frac{1}{d} \quad (4)$$

$$\text{" IV, } pG' = 10.72 - 0.90 \frac{1}{d} \quad (5)$$

$$\text{" V, } pG' = 4.66 - 0.90 \frac{1}{d} \quad (6)$$

In the case of the carboxyl dissociation of the mono-substituted amino acids, the value for the intercept constant, 4.83 (Curve III, Fig. 2) represents the value for the acid group when the amino group is removed to an infinite distance along the carbon chain. This value is characteristic for an unsubstituted long chain acid. Again in the case of the mono-substituted amino acids, the value for the intercept constant, 10.72 (Curve IV, Fig. 2) represents the dissociation of the amino group when the carboxyl group is removed an infinite distance. This value is characteristic of the aliphatic amines. It is evident, therefore, that in the case of these oppositely charged groups, that increasing the distance between them tends to eliminate the influence of that group which is substituted in the chain. On the other hand, with substituents of like

nature to the dissociating group, the value of this extrapolated constant fails to yield the value of the dissociation constant of the group unhindered by the presence of the substituent. Thus in the case of the dimethylene diamines, the value of the intercept constant is 11.26 (Curve II, Fig. 2), which is somewhat higher than the characteristic value of the aliphatic amines; in the case of the dicarboxylic acids this constant is 4.66, a value within the order of magnitude of the aliphatic acids but withal somewhat lower than the observed values; while in the case of the dissociation of the carboxyl group in the diamino acids the effect is complicated by the presence of not one but two amino groups.

It is to be expected as a result of these studies, that the dissociation constants of the proteins will be uninterpretable when taken on the basis of the corresponding values for similar groups in the constituent amino acids. Proteins containing an excess of basic groups will have their basic groups dissociate at reactions more acid than those characteristic of the free basic molecules—conversely, proteins possessing a considerable number of acid groups will have their acid groups dissociate at a more alkaline reaction than can be accounted for on the basis of the dissociation constants of the free acid groups. Whatever may be the more complex spatial relations of the free groups in the proteins, the general trend of the shifting of the values of such groups in proximity must be similar to that observed in the simpler substances presented in this communication.

#### SUMMARY

1. The titration constants have been determined of  $\alpha$ ,  $\beta$ -diaminopropionic acid. A comparison of these with the titration constants of  $\alpha$ ,  $\delta$ -diaminovalerianic acid and of  $\alpha$ ,  $\epsilon$ -diaminocaproic acid reveals a progressively acid character of the molecule as the distance between amino groups diminishes.

2. The MacInnes equation according to which the logarithms of the dissociation constants vary proportionately as the reciprocal of the number of carbon atoms separating the charged groups from each other has been employed to compare these diamino acids with substances of analogous constitution. Moreover, the constant of proportionality appears to be identical in the case of substances influenced by the same substituted group and independent of the nature of the ionizing group.

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## THE RACEMIZATION OF ACETYL-*L*-TRYPTOPHANE

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In the preparation of acetyltryptophane an unusual racemization was encountered which gives promise of yielding a general method for the racemization of amino acids. The advantages obtaining from such a method, in view of the difficulty of racemizing certain amino acids, are quite apparent. It need only be pointed out that if such a method were available the proof of structure of amino acids would be materially assisted. For instance, in the proof of structure of methionine such a method would have been of much value. The physical properties of the synthetic compound and its derivatives could have been compared with the racemized natural product. Otherwise resolution of the synthetic compound is necessary to obtain the active synthetic form for accurate comparison with the natural isomer. There are also many instances in which the isomer not occurring in nature is desired; and where the method of synthesis of the inactive compound for resolution purposes is very difficult, a convenient method of racemization of the natural isomer is highly desirable. In the present paper the racemization of *L*-tryptophane and the resolution of the resulting inactive amino acid will be presented.

In connection with another investigation that we were carrying out the acetyl derivative of *L*-tryptophane was desired. The compound was prepared by the Schotten-Bauman reaction, acetic anhydride being utilized according to the procedure used by Hollander and du Vigneaud (1) for the acetylation of cystine. In comparing the physical properties of the compound so prepared with those already given for acetyl-*L*-tryptophane in the literature (2), it was found that our compound had not only a different melt-



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ing point but also a different crystalline structure. It melted at 190° and crystallized in long, flat, blunt, needle-like crystals; whereas the compound described by Berg, Rose, and Marvel (2) melted at 206° and crystallized in glistening platelets.

The method of Berg was then followed and a product having identical crystalline form and melting point with the compound described by him was obtained. Both compounds yielded correct analyses for acetyltryptophane.

The two methods of preparation were very similar except that different proportions of acetic anhydride and sodium hydroxide were employed. In our method 2.54 mols of acetic anhydride and 5.82 mols of sodium hydroxide were used for 1 mol of tryptophane, while in the other procedure approximately 6 mols of acetic anhydride and only 2 mols of sodium hydroxide were employed for the same amount of tryptophane. It is evident that in the latter method there was an excess of acetic anhydride present compared to the amount of sodium hydroxide used, and that therefore the acetyltryptophane was in contact at 38° with acetic anhydride during a period of 3 hours, the time allowed for decomposition of the excess acetic anhydride.

The studies of Bergmann and Zervas (3) on the catalytic racemization of amino acids by acetic anhydride suggested to us the seemingly rather remote possibility that racemization might have occurred in the procedure used by Berg, which would explain the difference in the two acetylation products. For such a racemization to occur under these conditions, particularly in aqueous solution, by acetic anhydride would be surprising. Yet, this proved to be the case, for the acetyltryptophane prepared by our method had a rotation of  $[\alpha]_D^{31} = +29^\circ$ , whereas the other compound was optically inactive.

We were furthermore able to racemize our acetyltryptophane by subjecting it to the conditions of Berg's acetylation procedure by treating an aqueous solution of its sodium salt with the amount of acetic anhydride used in that method and allowing the mixture to stand at 38° for 3 hours. An optically inactive product was obtained identical with that obtained by Berg's method.

In order to place the proof upon an even more substantial basis the inactive acetyltryptophane prepared by Berg's method was resolved by means of the *d*-phenylethylamine salt. Acetyl-*d*-

tryptophane was obtained in this manner. When equal amounts of the acetyl-*d*-tryptophane so obtained and the acetyl-*L*-tryptophane made by our method were mixed and recrystallized, inactive acetyltryptophane, having a melting point and crystalline form identical with the original inactive acetyltryptophane prepared by Berg's method, was obtained.

The finding that the acetyl derivative of tryptophane fed by Berg, Rose, and Marvel (2) in their study of the utilization of various derivatives of tryptophane by the animal body was the racemic modification does not affect in any way the conclusion drawn by these authors that acetyltryptophane can be utilized for growth purposes, since they obtained a satisfactory growth response with the feeding of this derivative.

This unexpected racemization not only affords what we expect will be a fairly general method for racemizing amino acids but it also emphasizes the necessity for having sufficient sodium hydroxide present to decompose the excess acetic anhydride where this reagent is used in the Schotten-Bauman reaction for acetylating amino acids. There are a number of instances in the literature where this fact has not been appreciated. In a recent paper by Pirie (4) directions are given for the acetylation of cystine. It seemed very likely that at least some racemization might have occurred in this process. 50 cc. of 10 per cent sodium hydroxide and 16 cc. of acetic anhydride were used for 10 gm. of cystine. Pirie states that, "The excess acetic acid and acetic anhydride is removed by distillation at 40° *in vacuo* till a viscous gum remains." This might partially account for the low rotation of +0.42° that he found for the acetylcysteine. An acetylation of cystine carried out according to Pirie's directions convinced us that some racemization takes place under these conditions. In some preliminary experiments on cystine we found that under the acetylation conditions described above for preparing acetyl-*dl*-tryptophane, the sodium salt of acetyl-*L*-cystine is completely racemized, the reaction being accompanied by some decomposition as evidenced by the splitting out of some of the sulfur. This latter point is being more closely examined.

An extension of this racemization reaction to other amino acids is now being made, as well as a study of the mechanism of the reaction.

## EXPERIMENTAL

*Preparation of Acetyl-l-Tryptophane*

To 6.8 gm. of *l*-tryptophane dissolved in 10 cc. of water and 16.75 cc. of 2 N NaOH, 80 cc. of 2 N NaOH and 8 cc. of acetic anhydride were added in eight portions during an interval of about 15 minutes. The reaction mixture was kept in an ice bath during the process and the flask was vigorously shaken after each addition of the acetic anhydride. After the flask was allowed to stand at room temperature for 20 minutes, 35.80 cc. of 6 N H<sub>2</sub>SO<sub>4</sub> were added and the solution cooled in an ice-salt bath. The white crystalline compound which precipitated was filtered and then freed from any traces of tryptophane by washing with 25 cc. of 0.2 N HCl and finally with water. The compound crystallized from water in long, flat, blunt, needle-like crystals. A yield of 7.4 gm. of the recrystallized product melting at 189–190° (corrected) was obtained. The specific rotation of a 1 per cent solution in water in the presence of 1 equivalent of sodium hydroxide was  $[\alpha]_D^{31} = +29^\circ$ .

*Analysis*

3.307 mg. substance: 0.346 cc. N at 37.5° and 748 mm.

C<sub>13</sub>H<sub>14</sub>O<sub>2</sub>N<sub>2</sub>. Calculated. N, 11.38

Found. " 11.30

*Preparation of Acetyltryptophane According to the Method of Berg*

To 10 gm. of *l*-tryptophane dissolved in 100 cc. of 1 N NaOH, 30 cc. of redistilled acetic anhydride were added in 5 cc. portions, each addition being followed by vigorous shaking for several minutes. The solution was then allowed to remain at 35–40° for a period of 3 hours. A white precipitate of glistening platelets began to form. After the mixture was cooled in ice, it was filtered and the precipitate washed with cold water. The precipitate was then suspended in 0.2 N HCl, cooled, filtered, and washed until free from chlorides. 9.1 gm. of material melting at 204–205° were obtained, representing 75 per cent of the theoretical yield. After recrystallization the product melted at 205–206° (corrected). The compound was optically inactive.

*Analysis*

2.793 mg. substance: 0.292 cc. N at 37.5° and 748 mm.

C<sub>13</sub>H<sub>14</sub>O<sub>2</sub>N<sub>2</sub>. Calculated. N, 11.38

Found. " 11.30

*Racemization of Acetyl-L-Tryptophane*

2 gm. of acetyl-L-tryptophane prepared as described above were dissolved in 20 cc. of 1 N NaOH. To this solution were added 6 cc. of redistilled acetic anhydride in 2 cc. portions, the flask being vigorously shaken after each addition. The mixture was allowed to remain at 35–40° for 3 hours. At the end of this period considerable crystalline material had precipitated. The crystals had the same appearance as those obtained by Berg's acetylation method. After the solution had been cooled thoroughly in an ice bath the precipitate, consisting of glistening platelets, was filtered off and washed with cold water. 1.3 gm. were obtained. The product melted at 205–206° and a mixed melting point determination with the acetyltryptophane obtained by Berg's method showed no lowering of the melting point. The product was likewise optically inactive.

*Resolution of Acetyl-dl-Tryptophane*

The racemic acetyltryptophane was found to yield a nicely crystalline salt with *d*- $\alpha$ -phenylethylamine. For reference purposes in the resolution, the salt of acetyl-L-tryptophane was also prepared. 0.32 gm. of phenylethylamine and 0.68 gm. of acetyl-L-tryptophane were dissolved in 95 per cent ethyl alcohol and evaporated to dryness. The residue was then crystallized from alcohol, 0.34 gm. of the salt being obtained. The compound crystallized in needles and had a melting point of 192–194°. A 1.36 per cent solution in water gave a specific rotation of  $[\alpha]_D^{25} = +20.6^\circ$ .

To obtain some idea of the rotation of the phenylethylamine salt of the acetyl-dl-tryptophane in order to indicate approximately what the rotation of the salt of the dextro isomer might be, 2.200 gm. of acetyl-dl-tryptophane and 1.080 gm. of the phenylethylamine were dissolved in alcohol and evaporated to dryness. The specific rotation of the thoroughly mixed residue was  $[\alpha]_D^{25} = +2.1^\circ$ .

For the attempted resolution of the inactive derivative prepared by Berg's method 9.1 gm. of this compound and 4.46 gm. of phenylethylamine were each dissolved in a minimum of 95 per cent ethyl alcohol and then mixed and warmed. The total volume was about 50 cc. The mixture was then allowed to remain in the

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refrigerator for 7 hours, during which time a precipitate of long, silky needles formed. The yield was 11.5 gm. This product was recrystallized from 45 cc. of alcohol, 6.5 gm. being obtained. The third recrystallization yielded 4.6 gm. with a rotation of  $-19.6^\circ$ . Upon recrystallization the rotation remained unchanged. The compound had a melting point of  $194-196^\circ$ . The mother liquors were then worked up in the usual fashion and 1.1 gm. more of the pure phenylethylamine salt of acetyl-*d*-tryptophane were obtained.

*Analysis*

3.413 mg. substance: 0.346 cc. N at  $31.5^\circ$  and 753 mm.

$C_{22}H_{25}O_3N_2$ . Calculated. N, 11.45

Found. " 11.25

The free acetyl-*d*-tryptophane was obtained by treating the phenylethylamine salt with dilute sodium hydroxide and extracting the solution repeatedly with ether to remove the phenylethylamine. The solution was then neutralized with acetic acid, concentrated *in vacuo* to a small volume, acidified with sulfuric acid, and allowed to stand in an ice-salt mixture. Long, flat, blunt, needle-like crystals similar in appearance to the acetyl-*l*-tryptophane were obtained. The product melted at  $189-190^\circ$  (corrected) and had a specific rotation of  $[\alpha]_D^{32} = -30.2^\circ$ .

*Analysis*

3.016 mg. substance: 0.305 cc. N at  $30.5^\circ$  and 752 mm.

$C_{22}H_{24}O_3N_2$ . Calculated. N, 11.38

Found. " 11.25

Equal portions of the acetyl-*d*-tryptophane obtained from the above resolution of the inactive acetyltryptophane prepared by Berg's method and the acetyl-*l*-tryptophane prepared by our method from *l*-tryptophane were mixed and recrystallized from water. Glistening platelets were obtained identical in appearance with the original inactive acetyltryptophane prepared by Berg's method. The melting point of the product was  $204-205^\circ$  (corrected).

## SUMMARY

It has been demonstrated that an aqueous solution of the sodium salt of acetyltryptophane is racemized by acetic anhydride at  $35-40^\circ$  and that the product isolated by Berg in his acetylation of *l*-tryptophane is actually the acetyl-*dl*-tryptophane.

Acetyl-*dl*-tryptophane has been resolved by means of the *d*- $\alpha$ -phenylethylamine salt with the isolation of acetyl-*d*-tryptophane. Acetyl-*l*-tryptophane has also been prepared by the acetylation of *l*-tryptophane by the Schotten-Bauman reaction with acetic anhydride.

The necessity of having sufficient alkali present to decompose the excess acetic anhydride in a Schotten-Bauman reaction where acetic anhydride is used as the acetylating agent has been pointed out. Opportunity is otherwise afforded for racemization. Instances in the literature where insufficient alkali was employed have been indicated.

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# STUDIES ON MAGNESIUM DEFICIENCY IN ANIMALS

## I. SYMPTOMATOLOGY RESULTING FROM MAGNESIUM DEPRIVATION

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That magnesium is essential to animal economy is a proposition that has long been generally accepted without convincing demonstration. Apparently, the belief was grounded originally on the principle that the presence of a substance, particularly in considerable amount, in the body is purposive and constitutes *prima facie* evidence not only of its usefulness but also of its indispensability. Because of its plausibility, the lack of contrary evidence, the weight of authority, and the emphasis of repetition over years, this principle until recently passed unchallenged. Eventually, however, in the trend to supplant hypotheses with established facts, the validity of this teleological doctrine became conspicuously in need of verification. When it was subjected to investigation by experimental methods, the results in not a few instances were so at variance with the postulate that its soundness was seriously impaired. Specifically, the body was found to harbor so many adventitious substances that the necessity of such elements as magnesium, presumed solely on the basis of occurrence, was properly questioned.

Obviously, the doubt cast on the indispensability of magnesium could be settled most effectively, as has been done with other substances, by direct test. If it were possible to compose a synthetic diet, in which the purity of each ingredient was controlled so that magnesium could be added or omitted at will, that would be the ideal method. Such a procedure is unfortunately beyond

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present attainment. Another method, however, which is both satisfactory and workable, is available. In this method the relatively pure constituents of the ration, such as the mixture of salts, may be used after simple omission of magnesium compounds, or at most after a comparatively simple chemical treatment has freed the mixture from residual traces of the element; while the cruder components, such as protein and vitamin preparations, known to contain considerable magnesium, are subjected to such processes of purification as will reduce materially their magnesium content without seriously disturbing their physiological potency. Admittedly such a diet is not magnesium-free; but it may be sufficiently magnesium-poor to be below the animal's requirements. If animals cannot survive on a diet adequate in all respects except magnesium, the necessity of magnesium in animal physiology is thereby established. The success of the method depends upon the extent to which the magnesium may be removed from the ration without destroying the activity of the other ingredients.

Although the possibility of demonstrating the necessity of magnesium by means of a diet deficient in it must undoubtedly have been considered often in the past, it is interesting that a recent review (1) devoted to the rôle of magnesium in life processes mentioned only two attempts in this direction. In 1918, Osborne and Mendel (2) prepared a diet containing only 0.012 per cent (1 part in 8300) of magnesium. On this ration one rat of 60 gm. initial weight reached 340 gm. in 306 days when its growth was arrested. During the next 94 days its weight receded to 290 gm. When the animal was transferred at this point to the control diet, containing 0.08 per cent of magnesium, its weight returned to 335 gm. in 10 days, and remained at that level for 3 weeks, at the end of which time the experiment was terminated. Another animal produced substantially the same record. In drawing conclusions from their work, Osborne and Mendel expressed the view that the animal body could thrive and complete its growth on a diet low in magnesium. They did not attach significance to the failure in weight which set in after the completion of growth and which disappeared with addition of magnesium to the ration. Indeed, their results taken alone would scarcely justify the assertion that magnesium is essential to life and growth. But

in the light of later developments we may wonder whether their diet was not near the critical level in its magnesium content, and whether it did not hint that further reduction would disclose the necessity of the element.

Whatever may have been the implication of Osborne and Mendel's work, Leroy (3) advanced the status of the problem by refining the test diet to the point where it contained only 0.00103 per cent (1 part in 100,000) of magnesium. Given to white mice when they were 26 days old, the ration arrested their growth in 9 to 13 days and led to death in 24 to 35 days. On the other hand, the control ration, which was the basal diet with 0.023 per cent of added magnesium, permitted normal growth and development. Moreover, the change to this control ration after the animals had been depleted on the magnesium-low diet sharply checked their weight losses, permitted resumption of growth, and prolonged their lives in excellent condition for the 190 days that the experiment was continued. These results apparently placed magnesium on the list of essential elements.

In indicating the indispensability of magnesium, Leroy made no mention of any signs of ill effects characteristic of its deprivation, except failure of growth; consequently, the open assumption is that his animals met an uneventful death. There is, however, undeniable precedent for the belief that magnesium deficiency should exhibit a characteristic symptomatology. The impairment of certain systems in the body by the absence of the other dietary essentials is too well established to demand any detailed statement. In view of these circumstances, it is curious that such matters as the physiological rôle of magnesium and the effects of its deprivation have been ignored. Even Leroy's observations, limited as they were to two or three animals with an incomplete account of technique, have not been extended by more tests, particularly on a different species. These gaps in the knowledge of the physiological aspects of magnesium prompted us to seek the answers to the following two questions: (1) Is magnesium an indispensable element for body function; (2) if it is essential, what effect has deprivation of it upon the body?

The technique employed in the present study differed in only one respect from that used by the previous investigators; namely, the refinement of the magnesium-low diet was carried to the point

where it became almost magnesium-free. As in our former studies on the physiological rôle of different minerals (4), we had recourse to spectrographic analysis in order to study the distribution of magnesium in foodstuffs and to formulate a ration in which the various dietary essentials would contain as little magnesium as possible. Preliminary analysis showed that sources of carbohydrate, protein, and vitamins B and G were not available in a native form free from magnesium. The final details which we adopted in preparing a diet extremely low in magnesium, with particular emphasis upon those items likely to introduce a considerable amount of the element, are presented in a later section. It is sufficient to mention here that we have succeeded in preparing a diet adequate in all respects except magnesium, this element being present only to the extent of 1.8 parts per million.

When weaned rats weighing 35 to 45 gm. are placed on this magnesium-low diet, they pass through a spectacular series of events leading to an early and violent death. Within 3 to 5 days, average 4 days, all the exposed skin areas become vividly red from vasodilatation and hyperemia in the vascular bed. The reddened appearance of the animals becomes intensified until the 11th to 14th day, average 12th day, when it slowly subsides to be followed in turn by marked pallor and finally by slight cyanosis. In animals which are older and heavier when restricted to the diet, the red stage may reappear after it has once faded. During the hyperemic period the animals are extremely irritable and hyperexcitable as is evidenced by the readiness with which they are startled by slight noises or shadows. The hyperexcitability becomes progressively more pronounced until the 18th day, when any sudden disturbance throws the animal into fright that is followed by a convulsive seizure. Although the convulsive attack may appear as early as the 11th day, the more usual time is the 18th day, and by the 23rd day practically all animals have had a first seizure. The sudden onset of convulsions is a striking feature. The excitable animal, startled by sound, races at rapid speed in a wide circle until it finally falls on its side. The entire body of the animal is now rigid, with head stretched back, fore limbs extended at three upper joints and flexed at the metacarpophalangeal joint, and hind limbs extended backward (Fig. 1). So fixed are the jaws that often the tongue is perforated by the clenched teeth. The

skin presents a waxy appearance. All respiratory movements cease during the attack and return with relaxation of the musculature. Priapism may appear at this time and persist until death.

This stage of spasticity is succeeded by a period of relaxation lasting only a very short time. While still lying on its side, the animal exhibits twitchings in various regions, or paddles rapidly with all extremities. Coincident with this behavior, the animal's eyeballs become more prominent, the ears stiffen and project backward against the side of the head, and the fur stands erect. Then reappears a tonic spasm in which the rigid body assumes a typical position, with fore limbs pressed tightly against the thorax, fore paws clenched, and hind extremities extended. This spastic condition may give way to clonic contractions in which the fore limbs are alternately drawn to the chest and extended from the body. Next the animal may suddenly leap into the air, at the

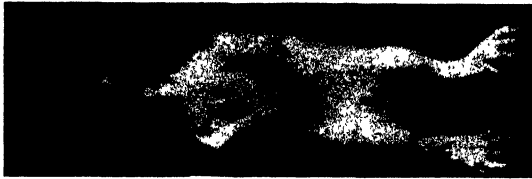


FIG 1. An animal, which had been deprived of magnesium, is shown in a state of rigidity which denotes the onset of convulsions.

same time spinning laterally several times; or it may "curl up" with marked flexion of all extremities; or it may do neither. There is marked cyanosis. Associated with the convulsive seizure is regurgitation of the stomach contents into the esophagus and mouth, as sacrifice experiments during this period have shown.

Within a short time the animal rears from the dorsal or lateral recumbent position in an attempt to stand, but its extremities will not support it. The animal buries its head in its outstretched fore limbs and propels itself forward entirely by its hind limbs, which, however, are so extended with paws hyperextended that the dorsal, not the plantar, surface bears the weight. Instead of forward motion, fine tremors may appear over the body. Throughout this stage the eyeballs are retracted.

Following the convulsive stage comes a recovery phase, doubt-

less dependent on exhaustion. During this period there is moderate cyanosis of skin, coldness of the extremities, lacrimation from dull, shrunken eyes, champing of the jaws, and drooling from the mouth. A hemorrhage may issue from the nose and orbit; and bloody, frothy fluid, consisting largely of regurgitated stomach contents mixed with blood, may bubble from the mouth. No urinary or fecal incontinence is seen during the attack.

If we arbitrarily demarcate the series of events, we may regard all the convulsions down to the recovery stage as one seizure. The first seizure usually lasts only a few minutes, and in the ensuing recovery phase a small percentage of the animals die. Most frequently one seizure follows another in rapid succession with short intervening recovery periods. All the successive seizures constitute an attack. A statistical analysis of the periods in which death occurred most commonly showed that 86 per cent of the animals died during the recovery phases of the first attack. Of this number, 7 per cent succumbed after the first seizure; while 93 per cent died after several seizures. The animals dying in the first attack had an average of five seizures.

The 14 per cent of animals surviving the first attack were free from convulsive disturbances for about 28 days before a second attack occurred. In this period priapism appeared among the males not previously affected. Usually the second attack of convulsions was characterized by four seizures, and 75 per cent of the surviving rats met death. Those animals left after the second attack were then again free from convulsive disorders for about 37 days. But eventually all animals were lost.

Since noises usually ignored by normal rats were sufficient to set off the convulsive attacks in the experimental animals—and in that sense the attacks were spontaneous—it is apparent that they may be induced intentionally during a susceptible period by similar stimuli. The hissing of a blast of air we have found to be the most effective means. After each seizure has been induced, it becomes progressively difficult to bring on the next, largely because of increasing exhaustion in the animal. Muscular fatigue may become so extreme that the animal cannot run; then, it is interesting to note that the exhausted animal responds to stimuli by slowly dragging itself around the same circular path over which it had previously raced. However, this slow motion performance

does not eventuate in a convulsion. Finally, a recovery stage is reached where it is impossible to initiate another seizure. The animal is greatly disturbed by the blast of air but can only emit a cry. Shortly thereafter such animals develop an unusual behavior. Selecting a spot in the side screening, they pace back and forth to it from the center of the cage. They snap as they wheel about in their pacing, until suddenly they sink their teeth into the wall of the cage and cling to it.

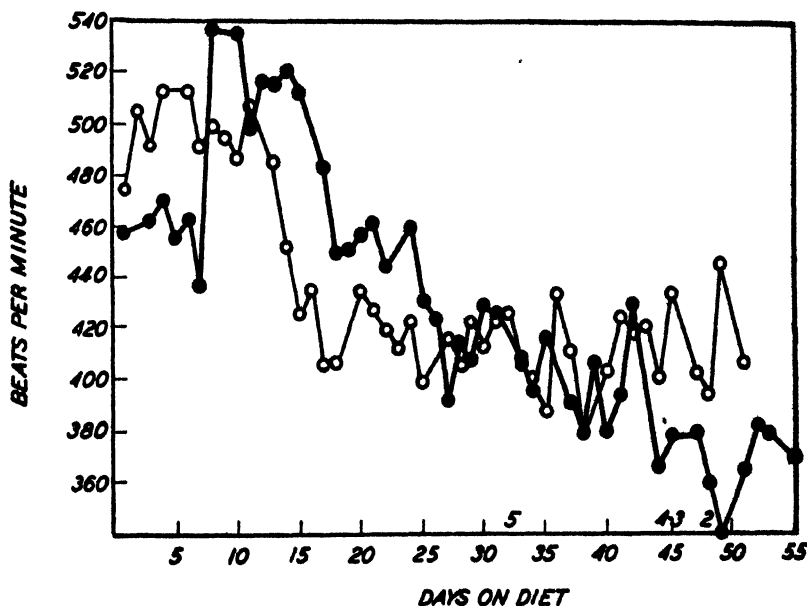


CHART I. Heart rates of animals on a magnesium-deficient diet as contrasted with those on a ration containing adequate magnesium. The curve with the solid circles indicates animals on a magnesium-deficient diet; that with the clear circles, normal control animals on a magnesium-deficient diet with added magnesium salt. The numerals above the abscissa indicate the number of experimental animals surviving at that period.

Throughout the prodromal period as well as during a seizure, daily heart rates of six rats on the magnesium-low diet were recorded by means of auscultation and an electric counter (5). Deviations from normal were ascertained by comparison with records obtained in the same manner for four control animals.

The daily rates for each group were averaged so that the results could be reduced to two curves (Chart I). Since the six animals in the magnesium-deficient group did not each survive for the same length of time, the number of individuals from which the average was drawn at various periods is designated on the chart. In the main, during the preconvulsive period the heart rates of magnesium-deficient animals differed from those of normal animals in two respects. With the appearance of the hyperemic stage the heart rate, which had been 460 to 470 per minute, increased to 535. After reaching this peak, it gradually fell coincidentally with the disappearance of the hyperemia and rejoined the plane of the nor-

TABLE I  
*Heart and Respiration Rates of Magnesium-Deficient Rats during a Convulsive Seizure*

♀ Black			♀ Black head		
Time	Respiratory rate	Heart rate	Time	Respiratory rate	Heart rate
<i>a.m.</i>			<i>a.m.</i>		
11.40	0	72	11.16	0	
11.41	92	180	11.19	0	88
11.42	108	216	11.20	40	80
11.43	116	252	11.22	0	
11.44	136	276	11.25	0	88
11.46	148	396	11.26	60	136
11.48	160	402	11.28	24	118
			11.29	0	
	Recovery		11.30	Death	

mal control animals. Just before the convulsive stage the curve of the magnesium-deficient animals is seen to deviate again from that of the normals; namely, the rate of the former became much slower, being reduced as much as 100 beats a minute. This retardation is not fully indicated by the curve, because the onset occurred in the rats at varying periods and a single lowering at an early date is masked by the rates of those animals as yet unaffected. The fall in rate is best seen by examining the records of individual animals. Associated with the bradycardia was marked arrhythmia.

In the seizures, the heart and respiration changes, which are

recorded in Table I, exhibit a behavior so often seen in convulsions. During the initial rigidity, the respiration ceases entirely, and the heart is markedly slowed, usually to 70 beats a minute. As the respiration gradually returns, the heart is swiftly regaining its usual rate, so that it precedes respiration in reaching the normal level. Ordinarily the heart has returned to its customary rate within 10 minutes after the onset of a seizure. In death, which always occurs in the recovery stage, the respiration while apparently recovering suddenly ceases. The heart keeps beating for a time, but finally it stops in ventricular systole.

In animals that have been deprived of magnesium for 4 to 6 weeks and have already survived the first convulsive attack, trophic disturbances make their appearance. Circumscribed lesions are seen in the skin in the form of erythematous, desquamated areas. Aside from general thinning of the coat, marked loss of hair occurs around the eyes, on the ears, under surfaces of the jaws, and neck. The nails show accentuated curvature, and are so brittle that they often break. In the ears, now pale, can be seen the residues of vascular accidents which occurred during the hyperemic stage; for old capillary breaks and even small hematomas are present. The paws and ears show evidences of edema. At the same time the urine is deeply pigmented, often being light brown in color. In a few cases blood was frankly present in the urine in the premortal stage; but usually the test for blood is negative.

Upon preliminary examination of the mouths of a limited number of animals, marked changes were apparent in the dental system.<sup>1</sup> The gums were swollen, hyperemic, and receding; often they showed slight lacerations. Pigmentation below the gum line was not uncommon in the incisors. In animals which had survived the 10th week on the magnesium-deficient diet, the peridental tissues were hypertrophied due to fibrous overgrowth; the molars and lower incisors were loose in their sockets; the upper incisors escaped. Whether these dental conditions will represent a constant finding cannot at present be stated.

Except for the dental findings, we have observed all the foregoing symptoms at various times in more than 200 animals. In

<sup>1</sup> We are indebted to Dr. Henry Klein for the pathological report on the teeth.



order that the symptomatology may be more definitely evaluated, we have compiled statistical data on 59 rats which were placed on the magnesium-low diet at an initial weight of 35 to 45 gm. Table II shows the relative frequency, time of onset, and duration of the symptoms, together with the mortality at different stages.

From Leroy's study as well as our own, we may conclude that the presence of symptoms and the time of their appearance depends

TABLE II

*Incidence, Time of Appearance and Duration of Symptoms, and Time of Death of 59 Rats Restricted at Initial Weight of 35 to 45 Gm. to a Magnesium-Low Diet*

	per cent	days
Vasodilatation.....	100	
Irritability and hyperexcitability..	100	
Convulsive attacks.....	100	
Onset of vasodilatation on 3rd day.	38	
" 4th	43	
" 5th	19	
Average time of onset of vasodilatation.		4
" duration of vasodilatation.....		8
Earliest appearance of convulsions.....		11
Average " " "		18
Mortality in first attack.....	86	
Mortality after 1 seizure.....	7	
" several (average 5) seizures.	93	
Survival of first attack.	14	
Survivors then free from symptoms.....		28
Mortality in second attack (usually with 4 seizures).....	75	
Survival of second attack.....	25	
Survivors then free from symptoms.		37
Mortality in third attack.....	100	

largely upon the extent of inadequacy of the diet in magnesium and upon the age and weight of the animals. We shall not attempt to explain Leroy's failure to record symptoms, but it does seem safe to assume that the much greater freedom of our diet from magnesium would lead to a more striking disturbance in the animal. As for age and weight, younger rats are more susceptible to magnesium deficiency than are older animals. The preceding account of the symptomatology was based upon the behavior of

animals of 35 to 45 gm. initial weight. When rats are confined to the magnesium-low diet at a weight of 50 to 60 gm., they reach the stages of vasodilatation and convulsions much later, and survive a greater number of seizures than do the younger animals. Some of these older animals particularly may pass through more than one stage of vasodilatation. The same prolongation of the

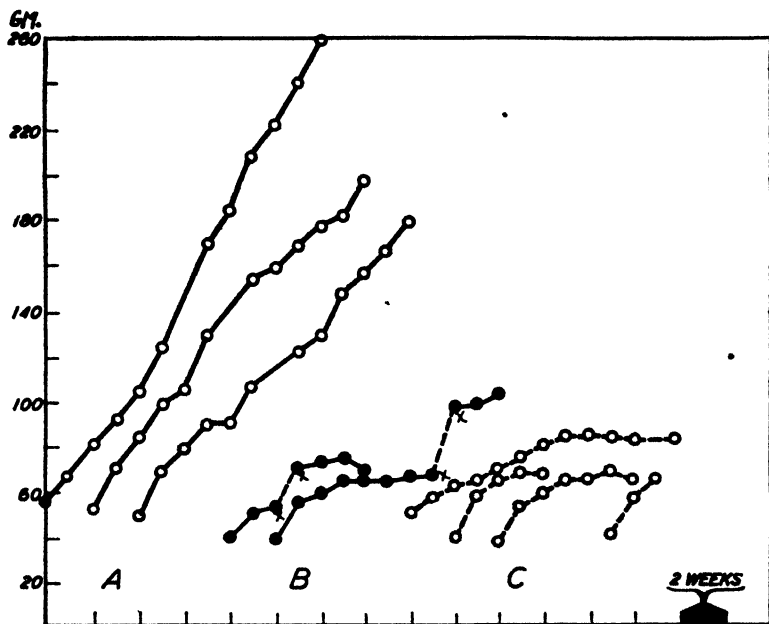


CHART II. Rat growth curves. A represents the growth of control animals fed the magnesium-low diet plus added  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ; B, experimental animals fed the magnesium-low diet except during the period X to X (indicated by the dash line) when they received control diet containing added  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ; C, experimental animals fed the magnesium-low diet throughout life.

survival period, only to a more marked degree, prevails in adult rats fed the magnesium-deficient ration.

That the symptomatology described in previous paragraphs is actually due to lack of magnesium and not to inanition or some unknown deficiency, is supported by three lines of evidence. Although the animals fed the magnesium-deficient diet do not show

optimum growth, their weight curves are fair. Often they are increasing rapidly in weight when taken off by a convulsion. If they survive an attack, they may increase in weight for a time but ultimately their growth is arrested. It is highly significant, however, that the animals usually die before loss of weight occurs. Typical growth curves are presented in Chart II, *C*. Further, throughout the life of the rat on a magnesium-poor diet no decrease in food consumption can be detected (Chart III). For these two reasons we believe that we can rule out inanition as a factor in the symptomatology of magnesium deficiency. Control experiments show that the behavior of animals on the magnesium-poor diet is specific for deficiency in magnesium and is not complicated by the lack of some unknown substance. When magnesium in the form of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  was restored to the diet of animals which had survived for

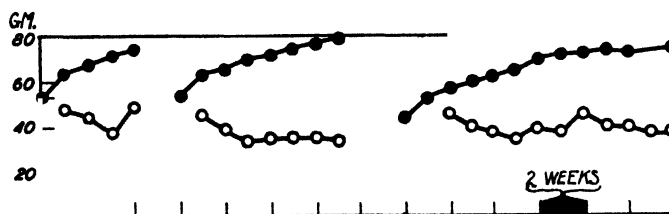


CHART III. Curves showing the growth of experimental animals on a magnesium-deficient diet and the corresponding food consumption records. Growth is indicated by the curves with solid circles; food consumption, by the clear circles.

4 weeks on the deficient ration, they gained 20 to 25 gm. in 5 days and showed a new growth of hair. Withdrawal of the added magnesium at this point immediately led to arrested growth, Chart II, *B*. Finally, animals fed the magnesium-low diet with added magnesium ( $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ) from the start showed excellent growth, normal appearance and behavior, and freedom of symptoms for 12 weeks, when the experiment was terminated (Chart II, *A*).

#### DISCUSSION

Even more than the remarkable behavior of animals when deprived of magnesium, the distinctive character and regularity in appearance of their symptoms argue for a specific syndrome, due

directly to magnesium deficiency or to the train of metabolic disorders following upon it. In attempting to analyze the symptoms and explain the underlying cause or circumstance of each, we must necessarily deal with probabilities until further lines of investigation actually establish the *modus operandi*. Pending that development, however, we feel it is not inappropriate to consider points that impress us as having a bearing on the pathogenesis of the disorder.

To mention only the outstanding phenomena, the hyperexcitability, vasodilatation, cardiac irregularity, generalized convulsions, and trophic disturbances require explanation. Of this group, the most striking is the vasodilatation. At first thought one would be inclined to link with this system an endocrine disturbance, involving particularly the adrenal, because this organ exerts influence over the distribution of blood in the body. Then, too, there is precedent for consideration of such a relationship between an endocrine organ and a chemical element, for instance, the thyroid and iodine, the parathyroid and calcium. But in view of the fact that the relationship in these familiar examples still abounds in controversial points, and a discussion of the physiology of the adrenal introduces, if anything, only more contention, it may be seen that postulation of a nexus between the adrenal and magnesium is not without difficulty in demonstration.

When the hyperexcitability and generalized convulsions are regarded in association with vasodilatation, the notion cannot be escaped that these symptoms may be part and parcel of one condition, tetany. In the familiar infantile tetany the cardinal feature is hyperirritability of the nervous system which may or may not lead to tonic spasms and generalized convulsions. The existence of hyperirritability characterizes the latent form which presents often no visible symptoms and is recognized only by artificial excitation of the peripheral nerves. Clinically, electrical reactions have been used for the detection of this condition, and have constituted the criteria for diagnosis. However, for three reasons we have not tested the electrical reactions in the rats on the magnesium-low diet. The animals are so hyperexcitable that no such sensitive method is needed. When, as we have repeatedly observed, noises that leave the control animals undisturbed throw the experimental animals into convulsions, it is evident that

stimuli other than electricity serve to indicate the extreme irritability of the nerves. Secondly, the irritable condition in our rats always leads to convulsions; *i.e.*, symptoms so readily recognizable that electrical excitation is not necessary. Finally, the growing distrust among investigators in the dependability of the electrical reactions seriously impairs their value. In citing the experience of many physiologists and clinicians who often found the reactions to produce discrepant results, Greenwald corroborates their view that reliance cannot be placed in the method (6). According to him, the reactions are dispensable.

If the condition induced in the magnesium-deficient animals is tetany, the question arises what is its type. In discussing the infantile form, Hess asserts, "One of the most valuable and constant signs of tetany is the lowered concentration of calcium in the blood" (7). From the close relationship known to exist between calcium and magnesium metabolism one wonders immediately whether magnesium deficiency manifests itself as a low calcium tetany. We are not yet prepared to comment upon the level of blood calcium in the experimental animals, but it is certain that a low calcium value is not necessary for production of tetany. Both on the basis of experience and theory, several independent factors may be said to determine the appearance of tetany. Hess lists various types of clinical and experimental tetany, together with the condition of the blood as respects calcium, phosphorus, and pH. In many instances the types were characterized by calcium values that were entirely normal.

In addition to the support from recorded evidence, strong theoretical considerations indicate that low blood calcium is not a *sine qua non* for tetany and that several factors may be responsible for the condition. From studies on isolated muscle, Loeb formulated the hypothesis "that irritability depends upon the various ions, especially the metal ions (Na, Ca, K, and Mg) existing in definite proportions in the tissues" (8). Carrying this concept even farther to the ultimate, Mathews adduced evidence to show that ions affect the nerves by virtue of their electrical charges; the anions increasing the irritability of motor nerves, while the cations act as depressants (9). He ascertained that the power of ions to stimulate or depress was the function of several factors, but principally the ionic potential; and that the effect of a salt upon a

nerve depended upon the net predominance in ionic potential of either the anion or cation. Now  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Ca}^{++}$ , and  $\text{Mg}^{++}$  are the usual cations found in the blood. Each has a different ionic potential. If the rationale of Loeb and Mathews be sound, it is not difficult to imagine that independent disturbance of any one of these ions would so shift the equilibrium of ionic potential as to increase the irritability of the nervous system.

Such considerations as these shed a different light on the nature of tetany. If its pathogenesis is not restricted to low blood calcium, if it is not infallibly diagnosed by electrical reactions, a revised view of its etiology and of the criteria for its recognition in animals is in order. As for its causation, we can do no better than quote Hess: "Tetany is not an entity from a pathogenetic point of view. . . . It is merely a symptom-complex resulting from and giving evidence of, an increased irritability of the nervous system and may be incited by a variety of factors. . . . Almost every chemical and physical agent in the tissues plays its part in determining the physiological status of the nerves." In regard to just what is meant by tetany in animals, a definition upon which its recognition can be based, Greenwald denotes it as "a spastic condition of the muscles, which is accompanied or followed by tremor, twitching, and tonic-clonic convulsions."

With these two concepts in mind, we may now apply them in determining whether it is reasonable to suppose that magnesium deficiency *per se* could lead to tetany, and particularly, whether the phenomena resulting from magnesium restriction really constitute tetany. The fact that low calcium tetany is the type seen most commonly in humans does not preclude the experimental production of tetany by other means. As a cation that normally exerts a depressant action on nerves, magnesium in the blood could conceivably be lowered to the point where it would turn the ionic potential in the direction of increased irritability of the nervous system, just as readily as does low blood calcium. As a matter of fact, magnesium possesses a somewhat higher ionic potential than calcium, so that deprivation of the former ion should disturb the electrical equilibrium at least as seriously as does lack of the latter ion. In this light, tetany becomes not merely a possible but rather a likely outcome of magnesium deprivation. Further, if the symptomatology of magnesium deficiency

be matched with Greenwald's conception of tetany, we find that the spasticity accompanied or followed by tremor, twitching, and tonic-clonic convulsions seen in our experimental animals fits his description.

When tetany is regarded as a syndrome producible by several means, it is interesting to consider certain differences in the manifestations of the type due to low calcium and that due to magnesium deficiency. In the former condition laryngospasm occurs with rather high frequency. Unless the cessation of respiration be interpreted as evidence to the contrary, true laryngospasm does not manifest itself in the animals receiving the magnesium-poor diet. On the other hand, the striking vasodilatation seen in the magnesium-deficient animals is not duplicated in low calcium animals, or in any other deficiency disease. This fact would seem to stamp the effects of magnesium deprivation as a specific condition, without, however, destroying its classification as tetany. Since the spasms of tetany vary in distribution, it is not impossible that in magnesium deficiency the vasodilatation is a manifestation of tetany, occurring as a vasomotor spasm and, like other kinds of spasms, appearing as a forerunner of generalized convulsions. The fact that the vasomotor mechanism is part of the vegetative system does not confute this point, because involvement of vegetative nerves in tetany has already been recorded. This concept regarding the vasomotor disturbance gains further support from Loeb's contention that various tissues must possess the several ions in different proportions, because each tissue has its own specific irritability. The implication is this: while being a non-specific condition due to the state of electrical potential, tetany will show different localizations of spasticity, dependent upon the different tissues containing varying proportions of the ions and what tissue is most affected by a change in concentration of a specific ion.

One more feature of the magnesium deficiency deserves mention. Whether the initially accelerated heart rate is due to hyperirritability of the nervous system cannot be said, but it is more likely that the vasodilatation is associated with a fall in blood pressure which in turn produces secondarily the more rapid heart action. That the heart rate returns to normal as vasodilatation disappears is a fact in harmony with this view. The progressive slowing of

the heart as the animal approaches the time for his initial convulsion is more difficult to explain, as is the arrhythmia associated with it.

Finally, the distinctive symptomatology, short life, and 100 per cent mortality of young rats deprived of magnesium appear to depend upon the extent of restriction of magnesium and the age of the animals. In Osborne and Mendel's experiments, where magnesium in the diet was reduced to 1 part in 8300, the animals reached maturity without signs of disturbance; while Leroy's animals (mice), receiving a ration containing 1 part of magnesium in 100,000, died in 24 to 35 days, apparently without symptoms. In our animals, restricted to a diet containing only 1.8 parts per million, a short and stormy course ensued, due perhaps to the extreme acuteness of the deficiency. Further, in general the younger the animal the shorter is its life and the more spectacular is its death. When animals of 55 to 60 gm. initial weight are deprived of magnesium, the appearance of vasodilatation is correspondingly prolonged, and the number surviving the first convulsive attack is greater than that among younger animals. But all eventually die. Probably the increased store of magnesium in the older animals is the deciding influence in the longer survival period.

Comparison of weight curves of our animals with those of the other investigators discloses a striking dissimilarity, doubtless due to differences in degree of magnesium deprivation. The rats in Osborne and Mendel's experiments showed good growth for more than 300 days, after which they failed slightly. The curves which Leroy exhibits for his mice are unusual in that they are parabolic. The animals thrived for a time, then ceased to grow, and finally failed to such an extent that they dropped to their initial weight before death. In sharp contrast to such records, the curves for our animals are still registering fair growth when suddenly terminated by fatal convulsions. Or if the animals survive for a considerable period, they may have their weight arrested but none shows appreciable loss before being overtaken by death. From evidence of maintenance of appetite, apparently anorexia and inanition play no part in magnesium deficiency.

In order that these data on the effects of magnesium deficiency may be amplified and the conclusions regarding the pathogenesis



may be verified, we have in progress studies from such angles as promise to yield the desired information. These studies include histopathology, blood chemistry, urine analyses, and electrocardiography.

### *Technique*

*Preparation of Magnesium-Low Diet*—Data obtained by spectrographic analyses showed that adequate diets, prepared from natural foodstuffs, invariably contained much magnesium. However, the analyses established that butter fat as a source of fat and vitamins A and E, viosterol as a source of vitamin D, and the mineral salts,  $\text{CaCO}_3$ ,  $\text{KCl}$ ,  $\text{NaCl}$ ,  $\text{NaHCO}_3$ , and  $\text{Fe}(\text{SO}_4)_3 \cdot (\text{NH}_4)_2\text{SO}_4 \cdot 24\text{H}_2\text{O}$ , were available in a magnesium-free condition; while sources of protein, carbohydrate, and vitamins B and G, were found to contain such amounts of magnesium as to require purification. Casein and starch were finally selected to furnish the protein and carbohydrate respectively in the diet, and the method of their refinement follows. Likewise, the details in preparing an extract of yeast, containing vitamins B and G, are given.

*Purification of Starch*—2 liters of 5 per cent  $\text{HCl}$ , by volume, are stirred thoroughly for 5 to 10 minutes into a pound of starch (Argo). After the starch has settled, the clear supernatant liquid is drawn off. The starch is then washed three times with distilled water, dried in a hot air oven, and finally pulverized.

*Purification of Casein*—Commercial casein, after being washed with a 0.2 per cent acetic acid solution and distilled water successively over a period of 10 days, is percolated in a Lloyd extractor with 70 and 95 per cent ethyl alcohol, each for 24 hours. The final product is dried rapidly under an electric fan.

*Vitamins B and G*—A 50 per cent alcoholic extract of yeast, prepared by treating 100 gm. of the yeast with 1 liter of solvent, is used to provide vitamins B and G, since an assay indicates its physiological potency as respects these two factors. Although this extract is not entirely free from magnesium, the amount of the element present is so slight as to make any further refinement inexpedient.

*Composition of Salt Mixture Used*

	gm.
CaCO <sub>3</sub> .....	1.5
KCl.....	1.0
NaHCO <sub>3</sub> .....	0.7
Fe <sub>2</sub> (SO <sub>4</sub> ) <sub>3</sub> ·(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> ·24H <sub>2</sub> O.....	0.508
KH <sub>2</sub> PO <sub>4</sub> .....	1.7
NaCl.....	0.5
Total.....	5.908

*Magnesium-Low Diet*

	per cent
Casein.....	20.0
Starch.....	56.1
Salts.....	5.9
Butter fat.....	8.0

50 per cent alcoholic yeast extract,\* 100 cc. in 100 gm. of ration; viosterol, 15 drops per kilo of ration.

This diet contains 1.8 p.p.m. (0.00018 per cent) of magnesium.

*Control Diet*

Addition of 0.05373 gm. of Mg in 100 gm. of diet.

	per cent
Casein.....	20.0
Starch.....	55.555
Salts.....	5.9
Butter fat.....	8.0
MgSO <sub>4</sub> ·7H <sub>2</sub> O.....	0.545

50 per cent alcoholic yeast extract, 100 cc. in 100 gm. of ration; viosterol, 15 drops per kilo of ration.

This diet contains 0.0539 per cent of magnesium.

The magnesium-low ration is made up in kilo lots. The dry components are well mixed with the butter fat, the yeast extract is then added, and the whole stirred thoroughly. The wet mixture is spread in trays which are exposed at room temperature to a current of air from electric fans until evaporation of the alcohol and water is complete. It is recommended that the diet be kept in a refrigerator to prevent the butter fat from becoming rancid.

The control diet is made up in the same manner with the exception of the addition of 0.05373 gm. of magnesium as MgSO<sub>4</sub>·7H<sub>2</sub>O to every 100 gm. of diet.

For our studies, ashes of these diets were prepared, and both

\* The amount of extract is approximately equivalent to 13.5 per cent of yeast.

spectrographic and chemical analyses were made to determine the magnesium content of the diets. These analyses were repeated at various times to check the magnesium content of the rations prepared at different times.

The magnesium content of the experimental diet is 1.8 p.p.m. or 0.00018 per cent of Mg and that of the control is 0.05391 per cent.

In our experiments all animals were confined to cages with screen bottoms, and distilled water was permitted *ad libitum*. Once weekly they received iodized water.

*Heart Rates*—While the animal is held by an attendant, the heart rate is determined by auscultation with a stethoscope in which a Y-tube replaces the usual bell. The auditor swings into the rhythm and reproduces it on a 2-way key connected in series with an electromagnetic counter. After the auditor has started dispatching the rate over an open circuit, another individual by means of a second key closes the circuit at will for a 15 second period. For our criteria, we demanded that two determinations 20 minutes apart on the same rat should check within 20 beats, else the count was repeated until satisfactory. It is appreciated that the method as outlined is not highly accurate, but practically it furnishes sufficient indication of actual conditions. For best results the animal should be held always by the same attendant.

#### SUMMARY

A diet containing only 1.8 parts per million of magnesium but otherwise adequate has been successfully prepared. When young rats are deprived of magnesium by restriction to this diet, they present a characteristic symptom complex which is terminated by early death. The symptom complex comprises vasodilatation, hyperirritability of the nervous system, cardiac arrhythmia, spasticity, and tonic-clonic convulsions. From a discussion of the pathogenesis and manifestations of tetany, it is suggested that the syndrome produced by magnesium deficiency constitutes tetany. Tetany induced by deprivation of magnesium possesses one symptomatic feature differentiating it from other known types; namely, the vasodilatation. Anorexia and inanition are shown not to be responsible for the syndrome. It is established that magnesium is an essential element for certain bodily activities, growth, and life.

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## THE MOLECULAR WEIGHT OF SPECIFIC POLY-SACCHARIDES\*

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The question of the molecular weight of the specific polysaccharides is one of great importance for an understanding of the mechanism of immune reactions. It has been found that the precipitin reaction, for example, may be accounted for as a chemical combination of antigen (or haptene) with antibody globulin in proportions depending on the relative amounts of antigen (haptene) and antibody present (1, 2). More particularly, the writers have shown (1) that in the presence of a large excess of antibody (*A*) the relative proportion of Type III pneumococcus specific polysaccharide (*S*) in the precipitate is as 1:120, and that the proportion increases to 1:60 at the "equilibrium point," at which the amount of *S* has been increased to the extent that the excess begins to appear in the supernatant fluid. The magnitude of these ratios is such that treatment of the reaction on a stoichiometric basis, or on the basis of the mass law, as was attempted by the writers, would be reasonable only if the molecular weight (formula weight) of *S* III were small in comparison with that of antibody globulin. Otherwise the precipitin reaction would be characterized as a colloidal, surface phenomenon, difficult of exact physicochemical analysis.

The assumption of a comparatively low formula weight for *S* appeared reasonable, especially as Dochez and Avery (3), the discoverers of the soluble specific substance of pneumococcus, had been impressed with its ready diffusibility through the body and its passage through the kidney. Likewise, its haptene nature,

\* The work described in this communication was carried out under the Harkness Research Fund of the Presbyterian Hospital, New York.

that is its ability to react with antibodies rather than to stimulate their production, indicated that it was but a fragment of the type-specific antigen of the intact pneumococcus cell (4). The greater speed of reaction of *S* with *A*, as compared with that of a protein with its antibody, is also in accord with the assumption of a smaller molecule for *S*.

Nevertheless, the first attempt to determine the molecular weight of *S* III has yielded a result of 118,000 at the hands of Babers and Goebel (5) who employed Northrop and Anson's diffusion method (6) and made their measurements only in 0.05 *M* phosphate solution. Information as to the molecular weight of *S* III cannot be obtained in this way, however, since by merely varying the concentration of salt values of the molecular weight, or rather, molecular volume, ranging from 360 to 541,000 may be obtained at will. The reason for this extraordinary behavior appears to be that *S* III is the salt of a strong, multi-valent acid (7, 8), and that, as shown in a recent communication of the writers (9), it is highly ionized in aqueous solution, giving rise to Coulomb forces of unusual magnitude. These forces increase with increasing dilution and render the diffusion method, or any other method for the determination of molecular weight based on the colligative properties of solutions, inapplicable without greater knowledge of the precise conditions under which fictitious values might be excluded and true figures found.

Especially in the case of *S* III, then, is it necessary to keep in mind the distinction between molecular weight as understood by the organic chemist (formula weight) and molecular weight as understood by the physicist (particle weight), since the formula weight may remain constant while the apparent particle weight may, and does in this instance, vary greatly. The diffusion method, when applicable, affords a measure of particle size, and the transition from particle size to formula weight is not always an easy one, as will be seen in the discussion. The present tendency, based on the x-ray studies of Sponsler (10) and Mark and Meyer (11) and the comprehensive work of Staudinger *et al.* (12) and of Carothers (13), is to regard molecules composed of long chains of simpler units as rod-like or thread-like in shape, and this has been clearly shown in the case of a polysaccharide such as cellulose. Since the calculations used in the diffusion method

involve the assumption of spherical particles, the question of molecular shape introduces an additional factor of uncertainty into the diffusion method as applied to polysaccharides.

It was hoped that Staudinger's recent development of a relation between viscosity and the molecular weight of polymeric compounds (12) might afford a solution of the problem, particularly as it had already been applied to a number of polysaccharides. The procedure involves (a) the preparation of a series of so called

TABLE I  
*Viscosities of S III Hydrolysis Products, Bingham Viscometer*

Fraction	Temperature	Concentration	Solvent	P (corrected)	Time	$\eta$	$\eta_{\text{solvent}}$	$\eta_r$	$\eta_{sp}$	$\frac{\eta_{sp}}{C}$
	$^{\circ}\text{C.}$	per cent		gm. per sq. cm.	sec.	centipoises	centipoises			
B-I	20	2.736	H <sub>2</sub> O	104.72	560.9	1.169	1.005	1.163	0.163	0.060
	20	2.736	"	198.48	295.6	1.167	1.005	1.162	0.162	0.059
	20	1.368	"	197.76	275.9	1.086	1.005	1.080	0.080	0.059
	30	2.736	"	104.62	445.3	0.927	0.801	1.158	0.158	0.058
	30	2.736	"	197.94	236.5	0.926	0.801	1.157	0.157	0.057
	20	1.368	0.17 M NaCl	213.34	258.1	1.096	1.008	1.087	0.087	0.064
B-II	20	1.368	0.17 " "	132.42	415.7	1.095	1.008	1.087	0.087	0.064
	20	2.949	H <sub>2</sub> O	195.31	293.8	1.142	1.005	1.137	0.137	0.047
	20	2.949	"	119.26	482.3	1.145	1.005	1.139	0.139	0.047
	20	1.475	"	110.81	490.0	1.081	1.005	1.075	0.075	0.051
	20	1.475	0.17 M NaCl	129.45	420.1	1.082	1.008	1.074	0.074	0.050
	20	1.475	0.17 " "	234.24	232.7	1.085	1.008	1.076	0.076	0.052
B-III	20	7.17	H <sub>2</sub> O	188.24	329.2	1.234	1.005	1.227	0.227	0.032
	20	7.17	"	106.24	535.2	1.237	1.005	1.231	0.231	0.032
	30	7.17	"	188.20	261.1	0.978	0.801	1.221	0.221	0.031
	30	7.17	"	106.05	463.7	0.979	0.801	1.222	0.222	0.031

polymer homologues, (b) the determination of the molecular weights and viscosities of the lower members of the series, (c) the calculation of a "molecular viscosity constant" for the series from these figures, and (d) the application of this constant to the viscosity figures obtained with the higher members, if these yield solutions which obey the Hagen-Poiseuille law; that is, if true molecular solutions are formed.

In the case of the Type III specific polysaccharide a partial polymer homologue series had already been described in the shape



of the aldobionic acid formed on energetic hydrolysis (7, 8) and several intermediate products formed under less drastic conditions (8). For present purposes the method of hydrolysis and isolation was modified in order to yield a series with a greater range of

TABLE II  
Calculation of Molecular Viscosity "Constants" ( $K_M$ )

Fraction	Mean $\frac{\eta_{sp}}{C}$ in H <sub>2</sub> O at 20°	Mol. wt. from reducing power	$K_M (\times 10^{-5})$ $(\frac{\eta_{sp}}{C \times \text{mol. wt.}})$	Specific volume (by Kunitz's formula)
B-I	0.059	1250	4.72	1.24
B-II	0.047	755	6.22	1.02
B-III	0.032	449	7.13	0.64

Molecular Weights Calculated from  $K_M$

Substance	Concentration	Solvent	$\frac{\eta_{sp}}{C}$	Mol. wt. calculated from		
				$K_M = 4.7 \times 10^{-5}$	$K_M = 7.1 \times 10^{-5}$	$K_M = 4.7 \times 10^{-5}$ corrected for hydration (formula weight)
S III	<i>per cent</i>					
	0.0147	H <sub>2</sub> O	29.35	625,000	413,000	1180
	0.0293	"	20.48	436,000	288,000	1280
	1.00	0.05 M Na <sub>2</sub> HPO <sub>4</sub>	3.28	69,000	46,000	2460
	0.10	0.05 " "	2.30	49,000	32,000	1070
	0.0293	0.17 " NaCl	2.17	46,000	31,000	960
	0.0293	1.34 " KCl	1.65	35,000	23,000	920
S I	0.0293	3.35 " "	0.64	13,600	9,000	910
	0.0131	H <sub>2</sub> O	6.12	130,000	86,000	930
	0.3273	0.17 M NaCl	1.28	27,000	18,000	
	0.3273	3.35 " KCl	0.90	19,000	13,000	1000
S II	1.106	H <sub>2</sub> O	0.20	4,300	2,800	1100
	0.553	0.85 M NaCl	0.13	2,800	1,800	980
S.G.A.	0.485	H <sub>2</sub> O	0.14	3,000	2,000	1000
B.B.G.	1.218	"	0.09	1,900	1,300	950

formula weights. Three fractions were thus obtained, one consisting of substances averaging 1250, a second averaging 755, and a third 449 in formula weight. These values were calculated from the iodine consumption of the terminal aldehyde groups, assuming one such group to a molecule, the only assumption justi-

fied by current views on the structure of polysaccharides. This application of the Willstätter-Schudel (14) method was first made by Bergmann and Machemer (15). The reducing groups were also determined by the Shaffer-Hartmann micro method (16) and the average of the two sets of values was taken. The molecular viscosity "constants" calculated from the data given in Table I ranged from  $4.7$  to  $7.1 \times 10^{-5}$  (Table II).

Due in part to failure to arrive at a molecular viscosity constant for the polymer homologue series, and in part to the exaggerated viscosity effects (9) connected with the unusual magnitude of the Coulomb forces engendered by the S III anion, it was not possible definitely to establish the molecular weight of S III by Staudinger's procedure. The extreme range of values obtained under varying conditions was, however, smaller than in the case of the diffusion method, and since the figures obtained permit several calculations of possible significance, the results are presented and discussed below.

Finally, a preliminary study was made of the diffusion of S III through a collodion membrane. While, in conformity with earlier observations (3, 7), S III does not diffuse through collodion into water, it is now shown that in the presence of 10 per cent sodium chloride on both sides of the membrane diffusion readily occurs.

#### EXPERIMENTAL

*Materials*—The S III used was prepared according to previous directions (7) with an additional purification by precipitation with barium hydroxide. A 2 per cent solution (by weight) was made up and dilutions were made from this by weight. The S III used contained 0.18 per cent ash, had an acid equivalent of 358, and showed  $[\alpha]_D = -34^\circ$  in 1 per cent solution.

The specific carbohydrate from the bovine tubercle bacillus (B. B. G.), prepared by Dr. A. E. O. Menzel in this laboratory, had an acid equivalent of 12,200, ash 0.11 per cent,  $[\alpha]_D = +92.8^\circ$ . It will be described in a later communication.

S I, S II, and specific gum arabic (S. G. A.) were prepared according to methods previously described (7, 17).

Sucrose and raffinose were obtained from commercial sources and were recrystallized from dilute ethyl alcohol.

*Determination of Diffusion Coefficients*—The rates of diffusion

of S III in water, 0.05 M  $\text{Na}_2\text{HPO}_4$ , and 1.71 M  $\text{NaCl}$ , of the non-acidic specific carbohydrate from the bovine tubercle bacillus in water and 0.05 M  $\text{Na}_2\text{HPO}_4$ , and of sucrose and raffinose in salt solutions were determined by the method of Northrop and Anson (6). The cells used consisted of a funnel provided with a stop-cock at one end and a Jena glass filter plate of porosity No. 7 fused on the other end. The diameter of Cells 1 and 2 was 28 mm., of Cell 3, 37 mm. The volumes were 24, 27, and 40 cc. respectively.

TABLE III  
*Determination of Cell Constants at 25° ( $\pm 0.5$ )*

Solvent, water.

Cell No.	Substance	Q cc. per day	Mean Q cc. per day	$D^*$ , cm. <sup>2</sup> per day	K
1	Sucrose, 0.25 M	0.809	0.815	0.428	0.53
		0.831			
		0.823			
		0.796			
2	" 0.25 "	0.762	0.759	0.428	0.56
		0.740			
		0.771			
		0.764			
2	Raffinose, 0.1 M	0.662	0.719	0.357	0.50
		0.708			
		0.776			
		0.728			
3	" 0.1 "	2.69	2.71	0.357	0.13
		2.73			
		2.77			
		2.66			

\* Extrapolated from Öholm's data (18).

The cells were standardized with sucrose or raffinose and the cell constants determined according to the relationship used by Northrop and Anson,  $D = K \frac{Q \text{ cc.}}{\text{days}}$ , in which  $D$  is the known rate of diffusion extrapolated from Öholm's data (18) and  $Q$  cc. is the number of cc. of the solution in the cell diffusing in a day. The results are given in Table III. 0.5 per cent phenol was added to all solutions to prevent bacterial growth, and the diffusions were carried out at  $25^\circ \pm 0.5^\circ$  in a constant temperature incubator.

In every case, except in the diffusion of S III in salt solutions, the concentration in the cell was corrected for the quantity of solute diffusing out during the period, the concentration used being the average of the concentration at the beginning and end of each diffusion period. The error involved in omitting this correction in the slow S III diffusion in salt was very small. The concentrations of sucrose, raffinose, and bovine tubercle bacillus polysaccharide in the cells and in the diffusates were determined from the optical rotation in 2 dm. tubes, with Hg green light. The quantity of S III diffusing was determined from the nitrogen precipitated

TABLE IV

*Diffusion of Sucrose and Raffinose in Salt Solutions at 25° (±0.5)*

Cell No.	Substance	Solvent	Q cc. per day	Mean Q cc. per day	K	D, cm. <sup>2</sup> per day	D*, cm. <sup>2</sup> per day, in water	D, cm. <sup>2</sup> per day, corrected for $\eta$ of solvent
1	Sucrose, 0.25 M	0.05 M Na <sub>2</sub> HPO <sub>4</sub>	0.778 0.796 0.807	0.794	0.53	0.42	0.43	
1	Sucrose, 0.5 M	1.71 M NaCl	0.586 0.600	0.593	0.53	0.31	0.41	0.36
1	Raffinose, 0.08 M	0.05 M Na <sub>2</sub> HPO <sub>4</sub>	0.660 0.660 0.660	0.660	0.53	0.35	0.36	
2	Raffinose, 0.08 M	1.71 M NaCl	0.557 0.536 0.558	0.550	0.50	0.28	0.36	0.33

\* Extrapolated from Öholm's data (18).

by an aliquot part of the diffusate from a standardized Type III pneumococcus antibody solution (19).

The radius of the particles was calculated from the equation

$$D = \frac{RT}{N} \times \frac{1}{6\pi r \eta} *$$

and the volume of a gm. molecular weight from the equation

$$\text{Volume} = \frac{4}{3} \pi r^3 N^*$$

\* For the derivation of the equation and definition of the symbols see (6).

For the sake of brevity only a summary of the data on sucrose and raffinose is given in Tables III and IV.

*Partial Hydrolysis of Type III Pneumococcus Specific Polysaccharide*—10 gm. of air-dry substance were dissolved in 50 cc. of 75 per cent (by weight) sulfuric acid immersed in ice water (*cf.* (8)). After standing overnight in the ice box, the solution was centrifuged to remove a small amount of undissolved material and the supernatant liquid was poured into a liter of cold water.<sup>1</sup> The solution was freed from sulfuric acid with barium hydroxide and the supernatant liquid and washings, containing the barium salts of the partially hydrolyzed polysaccharide, were concentrated to small volume *in vacuo*. An equal volume of a solution of barium hydroxide saturated at 60° was then added, this treatment being found necessary in some preparations in order to break up labile sulfuric acid esters of the polysaccharide formed during treatment with the strong acid. After standing overnight at room temperature, the mixture was centrifuged and the precipitate washed with saturated barium hydroxide solution. The supernatant liquid and washings were combined and the solution (Fraction II) and the precipitate (Fraction I) separately freed from barium with sulfuric acid and again converted into the neutral barium salts. Each solution was concentrated to small volume *in vacuo* and precipitated with alcohol. The precipitates were dissolved in water and fractionally precipitated with alcohol. Practically all of the material derived from Fraction I was insoluble in 10 per cent ethyl alcohol. The supernatant liquid from this was added to Fraction II which was almost completely precipitated by 25 per cent alcohol. Each fraction was redissolved in a small amount of water and refractionated. Two fractions were finally obtained, one precipitated by 10 per cent alcohol (Fraction B-I) and the other by 25 per cent alcohol (Fraction B-II). Addition of more alcohol to the supernatant liquid from Fraction B-II gave no additional precipitate. The barium salts were filtered off and dried to constant weight *in vacuo* at 60°. The barium content of the fractions was determined by ashing samples with sulfuric and nitric acids.

<sup>1</sup> Occasionally S III is still present and can be removed as a first fraction on precipitation of the barium salts with alcohol or with an excess of copper sulfate.

Fraction B-I. 83.3 mg. substance gave 26.1 mg.  $\text{BaSO}_4$ . Ba, 18.4 per cent.

Fraction B-II. 57.2 mg. substance gave 20.2 mg.  $\text{BaSO}_4$ . Ba, 20.8 per cent.

The reducing power of each fraction was determined by the Shaffer-Hartmann micro method (16) and the average molecular weight calculated from this under the assumptions that each fraction represented a mixture of polyaldobionic acids whose degree of polymerization did not vary greatly within the fraction, and that all the substances possessed a single terminal aldehyde group (*cf.* (7, 8, 15)).

Fraction B-I. 8.9 mg. equivalent to 1.11 mg. glucose. Reduction, 12.5 per cent; calculated to ash-free basis, 15.3 per cent. Mean mol. wt. =  $\frac{180}{0.153}$  = 1180, the nearest round number.

Fraction B-II. 4.4 mg. equivalent to 0.88 mg. glucose. Reduction, 20.0 per cent; calculated to ash-free basis, 25.3 per cent. Mean mol. wt. =  $\frac{180}{0.253}$  = 710, the nearest round number.

Weighed samples of each fraction were dissolved in water and converted into the sodium salts by adding the calculated amount of sodium sulfate. The precipitated barium sulfate was washed several times with water. The washings were added to the original supernatant liquids and the solutions made up to volume.

Fraction B-I. 1.6775 gm., containing 1.3680 gm. ash-free substance, made up to 50 cc. 5 cc. samples were taken for Willstätter-Schudel sugar determinations (14). Used 2.25, 2.16 cc. of 0.1 N iodine; blank, 0.15 cc. Reduction equivalent to 18.9, 18.1 mg. glucose = 13.8, 13.2; mean 13.6 per cent. Average mol. wt =  $\frac{180}{0.136}$  = 1320, with the same assumptions as before.

Fraction B-II. 1.8617 gm. containing 1.4745 gm. ash-free substance were made up to 50 cc. 5 cc. samples used 3.86, 3.81 cc. of 0.1 N iodine; blank 0.15 cc. Mean glucose equivalent, 33.2 mg. = 22.5 per cent. Average mol. wt. =  $\frac{180}{0.225}$  = 800.

Average of mol. wts. by both methods, Fraction I 1250, Fraction II 755.

A fraction of low molecular weight (Fraction B-III) was prepared by hydrolyzing the specific polysaccharide in 75 per cent (by weight) sulfuric acid at room temperature. After 48 hours a

reducing sugar determination on an aliquot part of the solution showed a reducing power equivalent to 46 per cent of glucose. The free acid was isolated over the barium and brucine salts. As a crystalline acid could not be obtained, the quantity in solution was determined by titration with sodium hydroxide, and calculated by assuming an acid equivalent of 350.

The solution required 2.08 cc. of  $N$  NaOH.  $2.08 \times 0.350 = 0.728$  gm. of substance. The volume was made up to 10 cc. and 1 cc. taken for the Willstätter-Schudel sugar determination. Required, 3.16 cc. of 0.1  $N$  iodine; blank, 0.15 cc. Glucose equivalent = 27.09 mg. = 37.1 per cent.

Average mol. wt. =  $\frac{180}{0.371} = 485$ .

0.5 cc. was made up to 50 cc., and 5 cc. samples were taken for Shaffer-Hartmann sugar determination. 3.64 mg. gave the glucose equivalent as 1.59 mg. or 43.7 per cent. Average mol. wt. =  $\frac{180}{0.437} = 412$ .

Average of mol. wts. by both methods, 449.

The viscosity measurements on the S III hydrolysis products were made with a Bingham viscometer at Lafayette College, as described in an earlier paper (9). The writers wish to thank Professor E. C. Bingham for his many courtesies in connection with the measurements.

*Diffusion of Type III Pneumococcus Specific Polysaccharide in 10 Per Cent Salt Solution*—Two collodion bags (A and B) were made in a test-tube, with 6 per cent parlodion-ether-alcohol-acetic acid mixture (20). 10 cc. of 0.015 per cent S III solution in 10 per cent aqueous sodium chloride were placed in Bag A and the bag was immersed in about 50 cc. of a salt solution of the same strength. A solution of *S* of the same concentration in water was placed in Bag B, and immersed in water. After 48 hours the outside solution was tested with a Type III pneumococcus anti-serum. The bags were then thoroughly rinsed inside and out with water and the experiment repeated, with Bag B in the salt solution and Bag A in water, in order to make sure that the positive test was not due to an undetected leak, or to undue differences in permeability. The results are shown below.

	Outside fluid, water*	Outside fluid, 10 per cent salt
Type III antiserum (1:3)....	Bag B, —	Bag A, ++
“ “ “ (1:3)....	“ A, —	“ B, ++

\* Salt equivalent to 1 per cent was added before testing with serum. Controls, including those in 10 per cent salt, were negative.

## DISCUSSION

The theoretical basis for the diffusion of non-spherical particles such as polysaccharides has not been worked out, so that the application of existing formulas, based on the assumption of spherical particles, may lead to erroneous results. Nevertheless, since the question has been raised by the publication of Babers and Goebel (5), a provisional estimate of the formula weight of a specific polysaccharide such as S III on the basis of the diffusion data might be made somewhat as follows: Bruins (21) and especially Hartley and Robinson (22) have also recently demonstrated the absurdly low figures obtained for the particle size of colloidal electrolytes in the absence of salts, as well as the great retardation of the diffusion rate caused by low concentrations of salts. They have ascribed the difficulty to the Coulomb forces engendered by the colloidal electrolyte, a conclusion reached independently by the present writers. In the case of a sodium salt of a slowly diffusing anion, in water, the rapidly diffusing sodium ions would tend to drag along the slower anion, an effect recognized by the earlier workers in this field, and corrected for by carrying out the diffusions in the presence of a salt, which would tend to equalize the number of small ions on both sides of the membrane. Although in most instances a relatively low concentration of salt is sufficient to bring the diffusion rate down to a constant value, it is shown in Table V that the apparent particle size of S III keeps on increasing as these low salt concentrations are exceeded. Since the hydration, as measured by the viscosity, decreases at the same time, it is probable that a portion of the effect is due to the unusual magnitude of the Coulomb forces engendered by the polyvalent S III anion (for a full discussion, see (9)).

It is evident from the data recorded in Tables III and IV that it matters little whether the diffusion of sucrose or raffinose be carried out in water, 0.05 M disodium phosphate solution, or 10 per cent sodium chloride solution, provided in the last instance correction be made for the viscosity of the strong salt solution. However, the retarding effect of both salts is enormously greater in the case of S III, in which electrostatic forces exercise a predominating rôle (Tables V and VII). Strangely enough, even 0.05 M phosphate greatly retards the diffusion of the almost non-acidic bovine tubercle bacillus polysaccharide, so that this effect introduces an



additional uncertainty into the calculation of the formula weight of a polysaccharide from the diffusion data (Tables VI and VII).

Now, according to Kunitz's empirical relation (23) it is possible to calculate hydration from viscosity. In the case of a 1 per cent

TABLE V  
*Diffusion of S III (Cell 1, Volume 24 Cc.  $t = 25^\circ (\pm 0.5)$ )*

Concentration in cell	Volume of diffusate	N precipitated*	S III	S III total	Q cc.	Time	Q cc. per day	K	D, cm. <sup>2</sup> per day
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Diffusion of S III in water

mg. per cc.	cc.	mg. per cc.	mg. per cc.	mg.		days			
9.02	100	1.12	0.082	8.2	0.91	1.14	0.80	0.53	0.42
8.71	100	0.94	0.063	6.3	0.72	1.03	0.70	0.53	0.37
8.47	100	0.79	0.051	5.1	0.60	0.78	0.77	0.53	0.41
8.24	100	0.91	0.061	6.1	0.74	1.00	0.74	0.53	0.39
Mean.....									0.40

Diffusion of S III in 0.05 M Na<sub>2</sub>HPO<sub>4</sub>

10.0	20	1.21	0.093	1.86	0.186	1.92	0.097	0.53	0.051
	20	0.93	0.063	1.26	0.126	1.09	0.116	0.53	0.061
	20	0.88	0.058	1.16	0.116	1.20	0.097	0.53	0.051
	20	1.19	0.090	1.80	0.180	1.90	0.095	0.53	0.050
	40	1.03	0.072	2.88	0.288	2.83	0.102	0.53	0.054
Mean.....									0.053

Diffusion of S III in 1.71 M NaCl

4.7	20	0.195†	0.0138	0.276	0.059	1.00	0.059	0.53	0.031
	20	0.243†	0.0175	0.350	0.074	1.21	0.061	0.53	0.032
	20	0.340†	0.0253	0.506	0.108	2.00	0.054	0.53	0.029
	20	0.173†	0.0122	0.244	0.052	1.00	0.052	0.53	0.028
Mean.....									0.030

\* From antibody Solution B31 (19).

† From antibody Solution B26 (19).

solution of S III in 0.05 M phosphate the Kunitz relation yields a value of 28 for the specific volume; that is, the volume occupied in solution by 1 gm. of the anhydrous solute ((9) Table VI). The

apparent molecular volume (155,700) divided by 28 would then give 5600 as the molecular weight of S III corrected for hydration;<sup>2</sup> in other words, the formula weight. However, even if the Kunitz

TABLE VI

*Diffusion of Bovine Tubercle Bacillus Polysaccharide. Cell 3, Volume 40 Cc.*

Concentration in cell	Volume of diffusate	$\alpha$ Hg green	Amount diffused	Q cc.	Time	Q cc. per day	K	D, cm. <sup>2</sup> per day
In water at 25° ( $\pm 0.5$ )								
mg per cc.	cc.		mg.		days			
25.84	15.5	0.50	34.6	1.34	1.03	1.30		
24.94	15.0	0.55	36.9	1.48	1.09	1.36		
23.22	15.0	0.52	34.9	1.50	1.12	1.34		
22.42	15.0	0.44	29.5	1.32	0.96	1.38		
Mean .....						1.35*	0.13	0.18
In 0.05 M Na <sub>2</sub> HPO <sub>4</sub>								
15.41	15.0	0.32	21.5	1.40	2.00	0.700		
14.76	15.0	0.45	30.2	2.05	2.96	0.692		
Mean .....						0.70	0.13	0.09

\* Discarding one value of 1.51.

TABLE VII

*Effect of Salts upon Apparent Molecular Size of Polysaccharides*

Substance	Solvent	D, cm. <sup>2</sup> per day	Molecular volume
Bovine tubercle bacillus polysaccharide	H <sub>2</sub> O	0.18	3,980
"	0.05 M Na <sub>2</sub> HPO <sub>4</sub>	0.09	31,800
S III	H <sub>2</sub> O	0.40	362
"	0.05 M Na <sub>2</sub> HPO <sub>4</sub>	0.053	155,700
"	1.71 " NaCl	0.030	540,800*

\* Corrected for  $\eta_r$  of solvent.

relation is valid, this figure would still be too high, owing to the retarding effect of phosphate on the diffusion of even a scarcely acidic

<sup>2</sup> Since the particle is largely water, specific gravity need not be considered.

polysaccharide, as shown above. If, on the other hand, the Coulomb forces due to the S III ion are not completely neutralized by 0.05 M disodium phosphate, as appears likely from an analysis of the viscosity<sup>3</sup> and diffusion data, 5600 would be too low. If one arbitrarily assumes these effects to balance, 5600 would be a more reasonable estimate of the formula weight than 118,000 as suggested by Babers and Goebel (5). Incidentally, the value given by these workers, corrected for hydration as above, becomes  $\frac{118,000}{1.77 \times 28}$  or 2400, the difference from the value reached above being possibly due to the different analytical methods used.

There is another possible method of calculating the formula weight of a polysaccharide from the diffusion data. Let us assume that, in the four cases considered in the present paper, the action of electrostatic forces is negligible upon sucrose and raffinose in water or in salt solutions, upon S III in 10 per cent sodium chloride solution, and upon the bovine bacillus polysaccharide in 0.05 M phosphate solution. The polysaccharide molecules will then be free to rotate equally in all directions about the center of the molecule. Let us assume that in so doing the sum total of the random motions will be, in effect, a sphere whose diameter will be the length of the molecule, and that it is the radius of this sphere which is measured when a polysaccharide is subjected to diffusion in the absence of electrostatic forces. In Table VIII are given the calculated values of the radius and molecular volume, the number of disaccharide units in the chain,  $\frac{r \times 2}{10.3}$ , (10.3 Å. being the mean length of the disaccharide unit cell of cellulose, as established by the work of Sponsler (10) and Mark and Meyer (11)),<sup>4</sup> and the formula weight calculated from the number of multiples of this unit. It will be observed that sucrose and raffinose give lengths corresponding to 1.9 and 2.3 hexose units, respectively, and their failure to show the length corresponding to the number of sugar units is indeed in accordance with structural considerations (25). In the case of the bovine tubercle bacillus polysaccharide, 4.5 di-

<sup>3</sup>  $\eta_{sp}$  of 0.5 per cent S III in 1.71 M NaCl solution, 1.17; in 0.05 M  $\text{Na}_2\text{HPO}_4$ , 1.37 (calculated for the latter from the  $\frac{\eta_{sp}}{C}$  column in Table III (9)).

<sup>4</sup> 10.4 Å. is given by Gonell for chitin (24).

saccharide units are indicated, corresponding to a formula weight of 1460. The indicated length of the S III chain is 12 (aldobionic acid) units, corresponding to a formula weight of 4100, in very satisfactory agreement with the value deduced in the preceding paragraphs from more standard considerations.

As to the application of the Staudinger method (12), it is seen (Table I) that, although the partial hydrolysis products of S III appear to obey the Hagen-Poiseuille law and to exhibit no association effects with changing temperature, the values of the molecular viscosity "constant" (Table II) are very far from constant, show-

TABLE VIII  
*Molecular Volume, Radius, Length, and Calculated Formula Weight of Polysaccharides*

Substance	Solvent	$D$ , cm <sup>2</sup> per day	Molecular volume	Radius	No. of disaccha- ride units in chain $\frac{r \times 2}{10.3}$	Calcu- lated formula weight
				Å.		
Sucrose .....	H <sub>2</sub> O	0.428*	296	4.88	0.94	320
Raffinose .....	"	0.357*	510	5.85	1.14	390
Bovine tubercle bacillus polysac- charide .....	0.05 M Na <sub>2</sub> HPO <sub>4</sub>	0.09	31,800	23.2	4.5	1460
S III .....	1.71 M NaCl	0.03	540,800†	59.7†	11.6	4100

\* Extrapolated from Öholm's data (18).

† Corrected for  $\eta_r$  of solvent, 1.17.

ing a marked decrease with increase in molecular weight. The difficulty, in this case as well, is perhaps associated with the higher valence of the anion in the fractions of higher molecular weight and the accompanying increase in hydration (Table II). For this reason the lowest value for  $K_M$ ,  $4.7 \times 10^{-5}$ , is probably too high, so that the apparent molecular weights calculated with its aid may be too low. While the extreme values range from 13,600 to 625,000, depending on the concentration and the amount of salt present, 49,000 is probably the highest value in the range in which the constant is applicable; namely, the range in which the Hagen-Poiseuille law is obeyed and no association is evident (see (9)

Table II). If these molecular weight (particle weight) values are corrected for hydration, as in the last column of Table II, they reduce to a remarkably constant value of about 1000, which might be taken as the formula weight of S III, as found by this method. Since this value is probably too low, as indicated above, the writers believe that the true formula weight will be found between the limits set by the two methods; namely, 1000 and 5600.

That S III is not diffusible through a collodion membrane in water but passes through in 10 per cent salt solution is now readily comprehensible on the basis of the unusually large Coulomb forces discussed above and in a previous paper (9) and may be taken as confirmation of a low formula weight for S III.

Table II brings out several additional points. The extreme variations in the particle weight of S I as calculated by the Staudinger method (with the same constant as for S III) are much smaller than in the case of S III, which is to be expected in view of the smaller viscosity anomalies observed in solutions of this substance, a behavior ascribed to the partial neutralization of the Coulomb forces arising from the anion by the basic groups present in the molecule (9). Moreover, the absolute values of the particle weights of S III, S I, S II, the specific gum arabic, and the bovine tubercle bacillus polysaccharide decrease with the decreasing acidity of the anions, indicating that in the Staudinger method, also, the charge on the anion cannot be neglected. In addition, all of the particle weights reduce to approximately 1000 when corrected for hydration. Although this value has no absolute significance for the reasons given above and since  $K_M$  is not necessarily the same for all the polysaccharides considered,<sup>5</sup> it may be taken as an indication that the molecular weights of the anhydrous substances (formula weights) are of the same order of magnitude and are probably less than 10,000.

Since all available evidence, including the corrected figure of Babers and Goebel, points to a low formula weight for S III, it is concluded that the ratios of 1:120 and 1:60 referred to as existing between S III and antibody in the precipitates formed by their interaction are consistent with a stoichiometrical or mass law

<sup>5</sup> Values of  $K_M$  given by Staudinger for polysaccharides such as inulin and cellulose acetates differ very little, however, when calculated to the same basis, from that used by the writers.

treatment of the precipitin reaction, the more so as the molecular weight of serum globulin, the serum fraction with which antibody is closely associated, is estimated as 104,000 to 175,000 (26, 27). The latter figure, at least, as calculated by Adair and Robinson, represents actual formula weight. The writers therefore feel encouraged to continue their study of immune reactions as phenomena subject to the laws of classical chemistry.

#### SUMMARY

1. The diffusion method is difficult to apply to the determination of the molecular weight of polysaccharides.

2. The determination of the molecular weight of S III, by diffusion or other methods, is complicated to an exceptional degree by the magnitude of the Coulomb forces engendered by its polyvalent, strongly acidic anion.

3. When these effects are partially corrected for by the Kunitz relation between viscosity and hydration, a provisional value of 5600 may be arrived at for the formula weight of S III by the diffusion method. The diffusion data are also calculated by a new method which does not involve a correction for hydration and which leads to a result of 4100.

4. The Staudinger method, involving the application of a molecular viscosity "constant," when corrected for hydration, leads to a value of 1000.

5. It is believed that the true value of the formula weight of S III will be found within these limits.

6. On the basis of the reasoning outlined the formula weights of the other specific polysaccharides considered should be less than 10,000.

7. The findings are consistent with the theory that immune reactions may be dealt with quantitatively according to the laws of classical chemistry.

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## STUDIES ON IRON METABOLISM AND THE INFLUENCE OF COPPER\*

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The investigations reported in this paper have been divided into three series of experiments.

*Series 1*—In Series 1 there were three groups of rats. In the first group (thirty-five rats) one rat was sacrificed every few days from birth on. The rats received milk alone after they were weaned. Those in the second group (eighteen rats) were given milk with an addition of 0.5 mg. of iron per day after they were weaned. Those in the third group (nine rats) received milk with the same addition of iron and 0.1 mg. of copper per day. Of the rats in the second and third groups one was sacrificed every few days from the time of weaning. The total iron in the bodies of the sacrificed rats was determined by the method given at the end of the paper. From the hemoglobin percentage and the weight of the rat determined just before the sacrifice of the animal, it is possible, by Chisolm's formula (1) which correlates weight and blood volume to calculate the total hemoglobin of the body. (The figures given by Scott and Barcroft (2) for the blood volumes of normal and anemic rats agree very well with the figures arrived at by Chisolm's formula.) Since hemoglobin contains 0.34 per cent of iron (3), it is a simple matter to calculate the amount of iron combined with the hemoglobin. The difference between the calculated "hemoglobin iron" and the total iron obtained by analysis gives a value for what has been called in this work the "non-hemoglobin iron."

\* The part of this work dealing with the effect of copper on the iron of the tissues was discussed at a meeting of the Society for Pediatric Research in March, 1929, and again in May, 1931. Neither discussion was published.



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From Chart 1 it may be seen that in those rats receiving the addition of iron or iron and copper the retention of iron averaged about 10 per cent of the intake, and that copper had no influence on the retention. The influence of copper, on the other hand, in

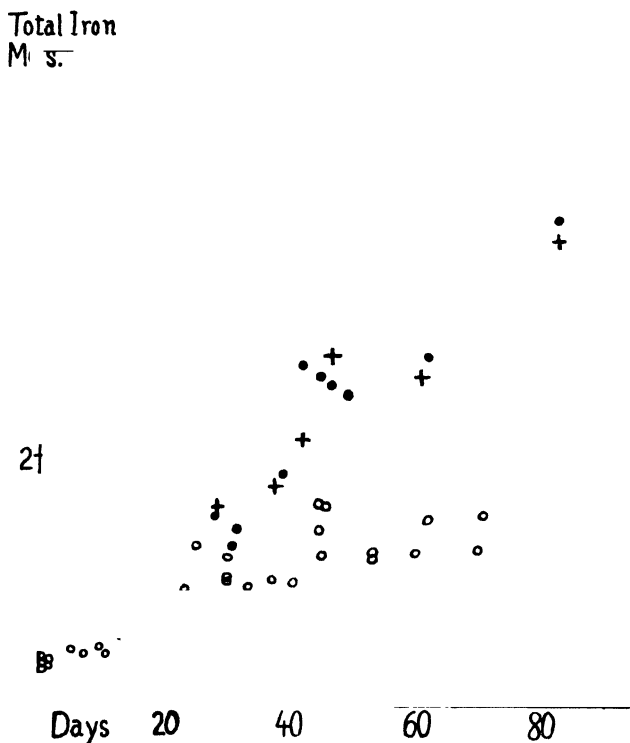


CHART 1. Total iron of rats charted against age in days. The clear circles refer to the rats on milk alone; solid circles, to rats on milk with addition of 0.5 mg. of iron daily; crosses, to rats on milk with the addition of 0.5 mg. of iron and 0.1 mg. of copper daily.

increasing the proportion of the retained iron found in the hemoglobin is evident from Chart 2. Chart 3 shows, as would naturally be expected, that those rats receiving iron and copper had smaller deposits of iron in the tissues (non-hemoglobin iron) than those receiving iron alone. An interesting fact is that the iron content of the tissues of the rats receiving copper in addition to iron is within the experimental error the same as that of the tissues

of rats receiving milk alone. This, of course, means that practically 100 per cent of the iron retention is used for hemoglobin under the influence of copper.

It must be noted that in these experiments it is quite possible, since the cages were of galvanized wire, that the animals may have ingested minute amounts of metals not added to the food, so that these experiments cannot be used to demonstrate that copper is not necessary for hemoglobin formation. Aside from the wire of

Hæmoglobin Iron

Mgs.

4|

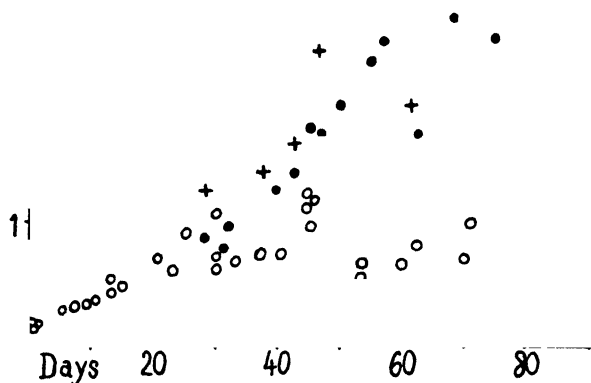


CHART 2. Hemoglobin iron of rats charted against age in days. For explanation of symbols see Chart 1.

the cages, there was, however, no source of extra metal: the milk was of "certified" quality, and the iron solution was made from iron wire (for analysis) dissolved in redistilled HCl.

Charts 4 and 5 have been prepared for a closer study of the iron retention and partition in rats, during the nursing period and later, on a minimal iron intake. From Chart 4 it may be seen that the curve of iron retention is nearly parallel with that of the gain in hemoglobin iron during the nursing period. After the

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nursing period is over, the hemoglobin iron tends to remain at a more or less constant level, while the very slow rise in total iron causes a slight divergence of the two curves. The relative constancy of the total hemoglobin content in the rats fed milk alone

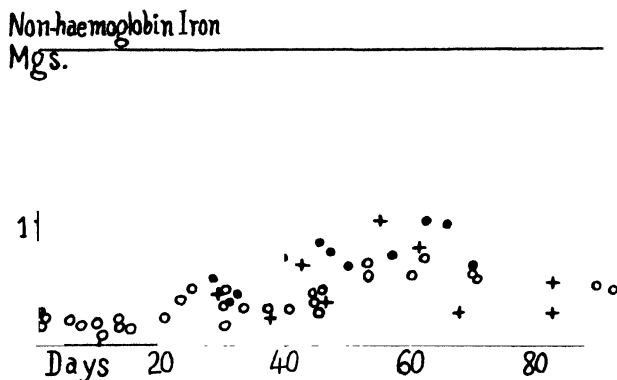


CHART 3. Non-hemoglobin iron of rats charted against age in days. For explanation of symbols see Chart 1.

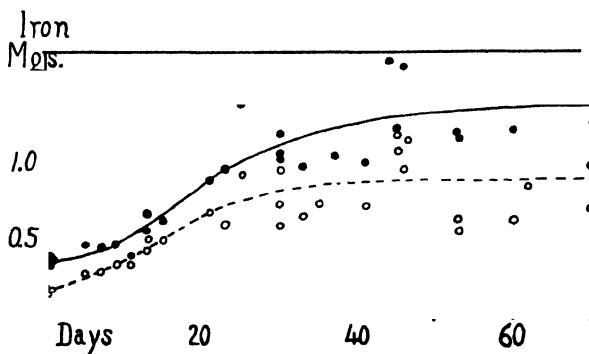


CHART 4. Total iron and hemoglobin iron of rats on milk alone charted against age in days. The solid circles refer to total iron; the clear circles, to hemoglobin iron.

has been noted in the great majority of animals in which hemoglobin determinations have been made at frequent intervals.

From Chart 5 it may be seen that the concentration of tissue iron reaches, after about the 30th day of life, a level below which it does not tend to go. The divergence of the two curves of Chart

4 is a measure of the increase of non-hemoglobin or tissue iron. This divergence becomes less and less and finally ceases at about the 60th day of life. It is noteworthy that the animals on milk alone in general failed to grow beyond this point. It is thus seen that the tissue iron was maintained at the necessary level first at the expense of the hemoglobin, since practically 100 per cent of the retained iron went to supply the tissues, then, when the hemoglobin concentration was reduced to its lowest point, the

Non-haemoglobin Iron

per cent  
4

24

14

Days      20                      40                      60                      80

CHART 5. Concentration of non-hemoglobin iron in rats fed milk alone

tissue iron was maintained at the expense of the growth of the animal. On the other hand, when iron was plentiful, 80 per cent of it went into hemoglobin formation, and this 80 per cent was raised to 100 per cent under the influence of copper. Although copper reduced the tissue iron, this reduction was not, in general, carried below the level of concentration found in Chart 5.

*Series 2*—The procedure was as follows: Litters of rats were fed on milk alone for a period varying from 5 to 58 days, the

preliminary period; then pairs from the same litter were selected on the basis of similarity of behavior (rate of growth, hemoglobin content, etc.); to the diet of one of the pair was added iron, to that of the other iron and copper. The amount of iron given varied between 0.5 and 1 mg. but each rat in a pair received the same amount. After the animals had been given iron or iron and copper for a second experimental period that varied from 15 to 48 days, they were sacrificed and the total iron determined by analysis.

In order to estimate the total iron of the rats at the beginning of the experimental period use was made of the constancy of the non-hemoglobin iron concentration after the 30th day of life in rats fed milk alone (Chart 5). From the value obtained from the chart, 0.5 mg. per 100 gm. of body weight, the non-hemoglobin iron content of the rat can be calculated according to the formula  $\frac{0.5 \times \text{weight (gm.)}}{100}$ . The hemoglobin iron is calculated as above

from the weight, and hemoglobin percentage by means of Chisolm's formula. The sum of the two is the total iron.

Since by the foregoing method we may estimate the total iron at the beginning of the experimental period and we know by analysis the total iron at the end, we have the data for an estimation of iron retention during this period. In order to test the accuracy of this estimation, a study of iron balance was carried out on two pairs of rats for 5 weeks. The retention determined by the direct method of iron balance was respectively 2.55 and 3.30 mg. The retention estimated by the indirect method of calculating the total iron at the beginning of the period of study and subtracting this calculated value from that determined by analysis at the end of the period was respectively 2.90 and 3.05 mg.

Table I contains the results from the experiments of Series 2. Retention varied for the most part between 10 and 20 per cent, and no correlation could be found between the retention and such factors as hemoglobin per cent at the beginning of the experimental period, duration of the preliminary period, or gain in weight. Copper did not affect the retention to a demonstrable degree. The greater utilization of the retained iron for hemoglobin formation and the consequent smaller deposition of iron in the tissues under the influence of copper are striking. It is interesting to note

TABLE I

*Iron Retention and Partition of Retained Iron between Tissues and Hemoglobin during Recovery from Anemia in Rats Receiving Iron or Iron and Copper in Addition to Milk*

Rat No.	Duration		Fe intake mg.	Cu	Total Fe			Hemoglobin Fe			Non-hemoglobin Fe		
	Period 1	Period 2			Period 1*	Period 2†	Gain	Period 1	Period 2	Gain	Period 1	Period 2	Gain
	days	days			mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
2- 1	58	48	24	0	1.50	4.30	2.8	0.92	2.89	2.0	0.58	1.41	+0.8
2- 3	58	48	24	+	1.45	4.56	3.1	0.85	4.79	3.9	0.60	0	-0.6
2- 2	31	32	30	0	1.64	6.63	5.0	1.19	3.02	1.8	0.45	3.61	+3.2
2- 6	31	32	30	+	1.29	4.57	3.3	0.82	3.91	3.1	0.47	0.66	+0.2
8- 4	19	34	10	0	1.52	2.83	1.3	1.05	2.31	1.3	0.47	0.52	+0.07
8- 6	19	34	10	+	1.31	2.93	1.6	0.85	2.92	2.1	0.46	0	-0.46
8- 5	19	20	10	0	1.16	2.05	1.9	0.78	1.60	0.8	0.38	0.45	+0.07
8- 8	19	20	10	0	1.45	3.60	2.1	1.02	2.65	1.6	0.43	0.95	+0.52
10- 7	5	17	8.5	0	1.46	2.97	1.5	1.09	2.07	1.0	0.37	0.90	+0.5
10- 8	5	17	8.5	+	1.47	2.46	1.0	1.09	1.84	0.8	0.38	0.62	+0.2
10- 1	38	16	8.0	+	1.32	1.95	0.6	1.89	1.77	0.9	0.43	0.18	-0.3
10- 6	38	16	8.0	0	1.66	3.26	1.6	1.19	2.52	1.3	0.47	0.74	+0.3
12- 1	27	35	38	+	1.11	4.25	3.1	0.75	3.16	2.4	0.36	1.09	+0.7
12- 3	27	35	38	0	1.40	5.95	4.5	0.99	3.20	2.2	0.41	2.76	+2.3
12- 2	27	21	10	0	1.23	4.26	3.0	0.85	2.65	1.8	0.38	1.61	+1.2
12- 4	27	35	38	0	1.32	5.10	3.8	0.92	3.06	2.1	0.40	2.04	+1.6
12- 5	27	35	38	+	1.14	4.65	3.5	0.78	4.05	3.3	0.36	0.60	+0.2
12- 6	27	21	10	0	1.16	2.30	1.1	0.78	1.39	0.6	0.38	0.91	+0.5
12- 7	27	21	10	+	1.14	3.66	2.5	0.78	2.55	1.8	0.36	1.11	+0.7
12- 8	27	21	10	0	1.01	4.20	3.2	0.68	2.75	2.1	0.33	1.45	+1.1
12- 9	27	35	38	+	1.11	5.80	4.7	0.75	4.76	4.0	0.36	1.04	+0.8
12-10	27	35	38	0	1.27	5.95	4.7	0.88	3.74	2.9	0.39	2.21	+1.8
21- 9	42	28	16	0	1.35	2.18	0.8	0.65	0.78	0.13	0.70	1.40	+0.7
21- 8	42	28	16	0	1.24	2.43	1.2	0.54	0.82	0.28	0.70	1.61	+0.9
21-10	42	28	16	+	1.28	2.40	1.1	0.58	1.80	1.2	0.70	0.60	-0.1
21- 4	42	28	16	0	1.33	2.95	1.6	0.78	1.22	0.4	0.55	1.73	+1.2
21- 6	42	28	16	0	1.27	3.20	1.9	0.71	1.22	0.5	0.56	1.98	+1.4
21- 3	42	28	16	+	1.34	4.45	3.1	0.82	3.47	2.7	0.52	0.98	+0.5

TABLE I—*Concluded*

The data (weight and hemoglobin per cent) from which the calculations were made have been omitted.

Periods 1 and 2 represent the preliminary and experimental periods respectively. During the preliminary period milk alone was given; during the experimental period, iron or iron and copper were added to the milk.

The iron intake is the total for the experimental period.

\* Figures are for total iron calculated as outlined in the text from the hemoglobin and weight at end of the preliminary period.

† Figures for total iron by analysis at the end of the experiment.

again that the concentration of iron in the tissues of the rats receiving copper is about the same as that in the tissues of the rats receiving milk alone.

It is obvious that the errors involved in the calculation used in this work are considerable and affect especially the figures for non-hemoglobin or tissue iron. It seemed, therefore, advisable to obtain figures for tissue iron by direct analysis of blood-free tissues. For this purpose the experiments of Series 3 were undertaken.

*Series 3*—Four groups of rats were studied: (1) sixteen rats on milk alone from the time they were weaned; (2) eight rats on milk with the addition of copper; (3) and (4) eight rats on milk alone for a preliminary period; to the diet of three iron was then added, and iron and copper to that of five rats.

The method of rendering the tissues blood-free was as follows: Heparin was injected into the heart of the anesthetized rat. Then, after about 5 minutes, the animal was killed by increasing the anesthesia. The thorax was rapidly opened and a needle connected with a reservoir of normal salt solution was inserted into the left ventricle and pushed up into the aorta. The salt solution was then allowed to flow and at the same time the inferior vena cava was cut just above the diaphragm. After 10 or 15 minutes of perfusion the vessels everywhere could be seen to be blood-free. Tissue analyses showed that perfusion beyond this point did not cause any further reduction in the iron content of the tissues.

Analyses of liver, spleen, and muscle showed that the liver was the only tissue that had an increase in iron concentration after the ingestion of iron. The muscle tissue as a whole contained the majority of the tissue iron, but the concentration was relatively low

and not influenced by iron intake. The total amount in the spleen was too small to be of much account in a study of iron metabolism and the variations in concentration were too wide for profitable study. Therefore, I have reported here only the analyses made on liver tissue. The increase of iron in the liver alone is probably explained by the fact that the iron was ingested and, therefore, would be removed from the circulation by the liver before reaching

Iron  
Mgs. per cent  
15+

10

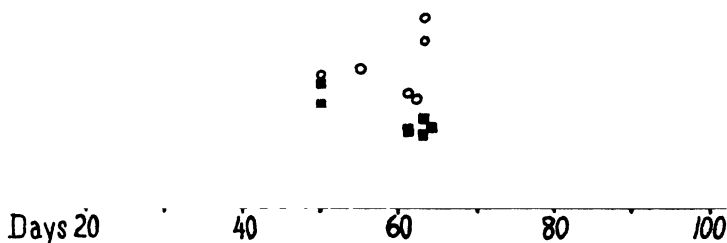


CHART 6. Concentration of iron in the blood-free liver. The clear circles refer to rats fed milk alone; solid squares, to rats fed milk with copper supplement; solid circles, to rats fed milk with iron supplement; crosses, to rats fed milk with iron and copper supplement.

the other organs. It is possible that the results would have been quite different had the iron been injected into the blood stream.

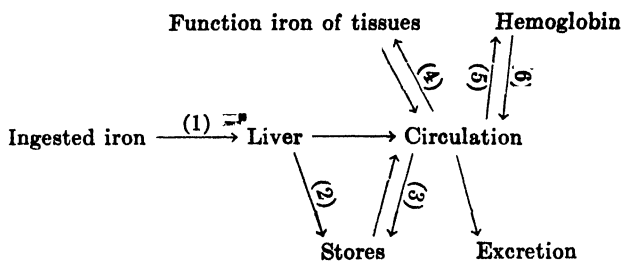
When the perfusion was finished, the liver was removed, dried, weighed dry, and ashed. Since it was desired to express the results in mg. per cent of moist weight, a small sample of liver was removed just before the perfusion was started for a determination of water content. This did not interfere in any way with the perfusion.



The results are contained in Chart 6. They confirm the facts: (1) that the concentration of tissue iron tends to be reduced under the influence of copper; (2) that the reduction is not carried beyond a certain point which is only slightly below that of the tissue iron in rats given milk alone; and (3) that when iron and copper were given together, the concentration of tissue iron is about the same as that in rats given milk alone.

*Comment*

The metabolism of iron may be pictured about as follows:



The ingested iron reaches the liver where it is rapidly removed from the circulation by the reticulo-endothelial cells. It may, however, reenter the circulation to be distributed as needed in the body or to be excreted. Breakdown of hemoglobin and of the cells of the body returns the "function" iron to the circulation, to be taken up again by the cells capable of storing it, or to be excreted. Thus, there is established within the body a kind of dynamic equilibrium which may be constantly changing; a sort of internal circulation which tends to conserve the body store of iron, so that the slight losses by excretion may easily be made up by fresh absorption. This scheme is, of course, no more at present than a working hypothesis, allowing us to picture and analyze the equilibria.

From the work reported in this paper we may divide the non-hemoglobin or tissue iron into two parts: a variable or mobile portion, and a fixed portion. Such a division has long been recognized histologically. The fixed portion does not tend to fall much below a concentration of 2 or 3 mg. per cent in the liver or 0.5 mg. per cent in the body as a whole, and this concentration is maintained at all costs. It is quite probable that this fixed iron corre-

sponds to the iron of the cell nucleus, possibly includes also the iron of muscle hemoglobin (the "function iron of tissues" in the schematic representation). Copper has no apparent influence on it. The level of concentration of "fixed" iron appears to be uninfluenced by the presence of an excess of iron, for, when copper and iron were given together, the concentration of tissue iron was not appreciably higher than when milk alone was given.

The mobile portion of the tissue iron forms the "stores" which from the time of Bunge have played such a large part in our understanding of iron metabolism. Bunge believed that the iron of the milk was altogether negligible in supplying iron for hemoglobin formation. With our present knowledge it is evident that the methods of that day were altogether too crude for a study of iron balance, but up to the present we have no adequate figures by which to know how far the iron present in milk can go toward satisfying the needs of the organism. From Chart 4 it seems that in the rat the amount of iron retained from the food in the nursing period is exactly the amount necessary for the formation of hemoglobin. Bunge did not take into account the function iron of the tissues, but it is just this small unconsidered quota of iron so necessary for the life of the animal that utilizes the stores as long as they are present, and later accounts for nearly 100 per cent of a minimal iron retention. As a matter of fact all the non-hemoglobin iron with which a rat is born would not suffice for more than 10 days of hemoglobin formation. Of course, the iron needs of the organism must be considered as a whole and from this point of view the iron retention from milk is inadequate for a growing animal. All that has been shown, however, is its inadequacy over a long period. During the nursing period the retention has not been shown to be insufficient.

It is the mobile portion of the non-hemoglobin iron that is influenced by copper. The effect of copper may be produced in one of three ways. A fourth way, *i.e.* by influencing iron retention, has been excluded from the possibilities. Copper may act at the arrows marked (2) and (3), preventing the cells that ordinarily store iron from taking it up or causing them to give it up if it is already there; in other words, changing the conditions of equilibrium in the direction of a decrease in the ordinarily great affinity of these cells for iron. It may act at the arrow marked (5), either

by generally stimulating the hemopoietic tissue, or by specifically increasing the rate of hemoglobin formation by catalytic action or other means. It may act at arrow (6) by decreasing the rate of hemoglobin breakdown. Nothing in the work reported here will help decide at which of these three points copper acts. Investigation has been undertaken to attempt to settle this question with material derived from the clinic. This work will be reported shortly.

#### *Method of Iron Analysis*

The rats to be sacrificed were killed by a blow on the head, care being taken not to lose any blood. The hair was burned and discarded. The abdomen was then opened; the gastrointestinal tract was removed, washed of its contents, and returned. The body was then dried, ground as completely as possible, and weighed. The grinding and mixing process was quite satisfactory except when the rat contained much fat. In this case it was necessary to make the sample for ashing larger. For the analysis, whole animals were taken only when the rat was very small—less than 10 gm. Otherwise, a sample was analyzed. The amount of the sample depended on the thoroughness of grinding and mixing, 2 or 3 gm. being sufficient when the mixing was satisfactory.

Ashing was done in platinum dishes in an electric furnace. The process was carried out gradually so that there should be a minimum of smoke. The ash was dissolved in about 20 cc. of  $N$   $HCl$ , transferred to volumetric flasks, and kept until the analysis was to be made. From 5 to 20 per cent of the total material was used for each analysis, according to the amount of iron expected, for the results were most satisfactory when not more than 0.1 mg. or less than 0.01 mg. was present. When the analyses were to be made, the solutions in the volumetric flasks were made up to volume and the sample pipetted into an Erlenmeyer flask. 5 cc. of  $N$   $HCl$  and 3 drops of  $HNO_3$  were added and the solution heated on a steam bath for at least half an hour. The analysis was then carried out colorimetrically by the thiocyanate method as follows:

The solution to be analyzed was cooled and transferred to a 50 cc. glass-stoppered cylinder. In a second cylinder were placed 5 cc. of  $N$   $HCl$ . 5 cc. of a 20 per cent  $NaSCN$  solution were added to each cylinder. The fluid in the two cylinders was adjusted to

about the same level with distilled water, and to the second cylinder was added a standard iron solution containing 0.02 mg. of Fe per cc., sufficient to bring the color to approximately the same point as in the first cylinder. (The standard solution was made by dissolving iron wire, for analysis, in HCl. The stock solution contained 10 gm. of Fe per liter. From this the solution used in the analysis was made up just before a series of analyses, N HCl being used for dilution.) 10 cc. of amyl alcohol were then added to each cylinder, and the cylinders shaken. The amyl alcohol layers were transferred to colorimeter cups and the comparison made.

#### SUMMARY

The retention of iron and its partition between the hemoglobin and the tissues has been studied in rats. It was found that during the nursing period the retained iron was sufficient to account for the gain in hemoglobin, but that, if a diet of milk was continued beyond the nursing period, all the retained iron went to maintaining the tissue iron at a constant minimal concentration which is considered to represent the "function iron" of the tissues. When extra iron was given, this was divided between the hemoglobin and the tissues, most of it going to hemoglobin formation. When copper was given, a larger proportion of the retained iron went to form hemoglobin, the tissue iron was reduced in amount, and the reduction was carried down to, but not below the level of concentration which was considered to represent the function iron of the tissues. Copper had no effect on iron retention. The distribution of iron in the body was diagrammatically represented, and discussed.

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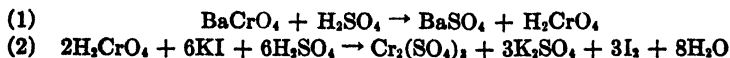
# AN IODOMETRIC MICRO METHOD FOR THE DETERMINATION OF SULFATES IN BIOLOGICAL MATERIAL

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For the quantitative determination of sulfates two principal procedures are available, the precipitation as barium or as benzidine sulfate. The former is still to be regarded as the method of choice from the standpoint of accuracy, but being a gravimetric procedure it requires fairly large quantities, unless the facilities are available for microanalytical gravimetric work. It seemed to us that the classical reaction of precipitation of the sulfate as  $\text{BaSO}_4$  could be retained but the estimation carried out volumetrically, thus making it suitable for microchemical analysis. It is a familiar fact that sulfates may be precipitated with barium chromate as well as barium chloride. Barium chromate dissolved in dilute hydrochloric acid reacts with  $\text{SO}_4$  ions giving a precipitate of  $\text{BaSO}_4$  and an equivalent amount of  $\text{H}_2\text{CrO}_4$ , which can be determined iodometrically by titration with thiosulfate. The fact that the  $\text{BaCrO}_4$  is dissolved in  $\text{HCl}$  is most favorable for the precipitation of the  $\text{BaSO}_4$ , and the excess  $\text{BaCrO}_4$  can be subsequently removed by making the mixture alkaline. Both the  $\text{BaSO}_4$  and  $\text{BaCrO}_4$  precipitates are removed by centrifuging, and aliquots of the supernatant fluid containing the  $\text{H}_2\text{CrO}_4$  after adding potassium iodide and sulfuric acid, are titrated with standard sodium thiosulfate solution. From the reactions involved



it follows that one I is equivalent to one-third  $\text{H}_2\text{CrO}_4$  and consequently to one-third S; or that 1 cc. of  $\text{N Na}_2\text{S}_2\text{O}_3$  corresponds to  $\frac{32}{3}$  mg. of S. The actual titrations can be made with 0.001 or

0.005 N  $\text{Na}_2\text{S}_2\text{O}_3$ , so that each cc. of thiosulfate used corresponds to 0.01066 or 0.0533 mg. of S, respectively. We carry out the titration with a micro burette so calibrated that the volume can be accurately estimated to the third decimal place. Furthermore, the aliquot used for the titration may be greatly varied also, either the entire amount of the supernatant fluid or only a small part of it being used for the titration, which gives the method still greater flexibility.

In the manner outlined above the method is applicable only to phosphate-free solutions of inorganic sulfates, the analysis of which can be carried out with great accuracy and rapidity. The routine procedure, as we have developed it, consists in measuring the unknown sulfate solution into a tube, 5 cc. of the solution being used, or diluting the volume to 5 cc. with water, when smaller quantities have been taken, and adding to this 5 cc. of the  $\text{BaCrO}_4$  reagent. Our reagent can be used safely with quantities of sulfate containing up to 1 mg. of sulfur. After allowing this to stand for 15 minutes 5 cc. of dilute ammonia are added, the mixture is well shaken, transferred to a centrifuge tube, and the precipitate thrown down by centrifuging for about 2 minutes. One may also use dry  $\text{Ca}(\text{OH})_2$  instead of ammonia to alkalinize the solution and thus retain the volume of only 10 cc. The supernatant fluid varies in color from a very pale to a fairly strong yellow, depending upon the amount of sulfate precipitated. We thought at first that the color due to the chromic acid could be utilized for a colorimetric procedure, but we found that it was not suitable for this purpose, even though in certain concentrations the color intensity did bear a fairly close relationship to the quantity of sulfate analyzed. However, the results were usually erratic and we gave this up entirely. Generally a 5 or 3 cc. aliquot, depending upon the intensity of the yellow color of the supernatant fluid, is taken for the titration. Working with known sulfate solutions of varying S content, we invariably found that the titration results plotted against the quantity of sulfur give a perfect straight line curve. We also found it much more desirable to standardize the determination with known and variable quantities of sulfate, calculating the factor for converting cc. of thiosulfate to mg. of sulfur, rather than running blanks and correcting the titration. The standardization can be carried out easily within an hour's time. One can

determine as many points as he wishes, although the determination on three or four known amounts is all that is really necessary.

*Determination of Sulfates and Sulfur Partitioning in Human Urine*

In this very simple, quick, and reliable form the method is unfortunately not applicable to the determination of the various sulfates in biological materials. It can be used, however, for the total sulfur determination, where, owing to the complete ashing and oxidation, the residue, of course, behaves like any inorganic solution. For the inorganic sulfates the method is not directly applicable to biological material, which must be subjected to a preliminary treatment. It must be stated that in this respect the method is more limited in its usefulness. We have worked out the conditions under which it is possible to employ it for the sulfate fractionation in human urine. In the first place, phosphates likewise form an insoluble salt with barium and release  $\text{H}_2\text{CrO}_4$  from the reagent. The phosphates must, therefore, be removed; otherwise the results will be too high. Secondly, the chromic acid, being an oxidizing agent, can react with reducing substances; and the extent to which this occurs tends to lower the results more or less. In urine we found that the uric acid interferes most seriously with the reaction. Urine must, therefore, be freed of both phosphates and uric acid before the sulfate determination can be carried out by precipitation with barium chromate. The first problem was easily and satisfactorily solved by treating the urine with an excess of dry  $\text{Ca}(\text{OH})_2$ . The quantitative removal of the uric acid was more difficult to achieve. Although it could be quantitatively removed by precipitation with  $\text{Zn}(\text{OH})_2$  or by adsorption with Lloyd's reagent (specially treated for our purpose), either method led to the complete removal of the sulfates as well. We finally made use of Ohta's (1) procedure for the destruction of the uric acid by hydrogen peroxide in the presence of ferric chloride, which likewise oxidizes other mildly reducing substances.

The method for human urine, as we finally developed it, is as follows:

*Reagents*

0.02 N barium chromate solution in 0.2 N HCl made by dissolving 2.53 gm. of the substance in 100 cc. of 2 N HCl and diluting to 1 liter.



Ammonia diluted 1:5.

Sodium thiosulfate, 0.001 N or 0.005 N solution.

0.01 N biiodate solution, for standardizing the thiosulfate.

Starch solution, 1 per cent in saturated NaCl.

Merck's superoxol, redistilled.

Dry, solid calcium hydroxide.

Manganese dioxide, highest purity.

Ferric chloride, 1 per cent.

Potassium iodide, 5 per cent.

10 N sulfuric acid.

*Procedure*—Urine was shaken with dry  $\text{Ca}(\text{OH})_2$  and after standing for an hour, filtered. It is advisable, though not at all essential, to pass  $\text{CO}_2$  to remove the excess calcium, and filter again. A sample of the phosphate-free urine filtrate, usually 2 cc., is titrated with 0.1 N HCl to a pH between 6 and 7, with alizarin as indicator. This generally requires about 0.5 cc. A 2 cc. sample of the urine filtrate is measured into a 160  $\times$  20 mm. Pyrex tube calibrated for 10 cc., and is acidified with the amount of 0.1 N HCl determined in the previous test. We found that adding the indicator directly to the tube in which the sulfate determination is to be carried out gave erratic results. A drop of 1 per cent  $\text{FeCl}_3$  is now added to the slightly acidified urine filtrate and the volume is diluted with water to about 5 cc. The tube is suspended over an electric heater and the contents are kept boiling *gently* for 10 minutes. During the boiling a few drops of superoxol are added from time to time, a total of 5 to 10 drops being used. At the end of this time the tube is removed from the burner and a pinch of  $\text{MnO}_2$  is thrown in to decompose the excess  $\text{H}_2\text{O}_2$ . The boiling is resumed and when the frothing ceases the reaction has come to an end. On cooling, 1 cc. of 0.1 N NaOH is added, which is sufficient to make the contents of the tube alkaline and to precipitate the iron and manganese; the volume is made up to 10 cc. with water, and the solution is filtered through an ashless filter paper (Whatman, No. 44). The filtrate is then made acid by adding a drop of concentrated HCl. A 5 cc. aliquot (equal to 1 cc. of the original urine) is measured into a tube, the sulfates are precipitated by adding 5 cc. of the  $\text{BaCrO}_4$  reagent, and, after standing 15 minutes, the contents are made alkaline by the addition of 5 cc. of dilute ammonia or of solid  $\text{Ca}(\text{OH})_2$  and well mixed. All the reagents

are measured with standard pipettes. The solution is centrifuged for 2 minutes, and 3 or 5 cc. aliquots of the supernatant liquid are used for the titration. These are measured into small Erlenmeyer flasks, treated with 1 cc. of 5 per cent KI, then with 2 cc. of 10 N  $\text{H}_2\text{SO}_4$ , and titrated immediately with 0.005 N (or 0.001 N)  $\text{Na}_2\text{S}_2\text{O}_3$ . The number of cc. required in the titration, corrected by the normality factor, and multiplied by the sulfur factor, gives the mg. of sulfur in the aliquot analyzed, or

$$\text{Cc. } 0.005 \text{ N } \text{Na}_2\text{S}_2\text{O}_3 \times \text{S factor} \times 5 \text{ (or 3)} = \text{mg. S per cc. urine}$$

To determine the total sulfates (*i.e.*, inorganic and ethereal), a 10 cc. sample of the original urine filtrate is measured into the 160  $\times$  20 mm. Pyrex tube calibrated for 10 cc. and, on adding 2 cc. of concentrated HCl, is boiled gently for 20 to 30 minutes. After cooling, the solution is made slightly alkaline with NaOH, a small piece of litmus paper being used as indicator, and diluted to the 10 cc. mark. The sulfate determination is again carried out on 2 cc. aliquots, exactly as was done previously for the inorganic sulfate determination. We have naturally tried to perform the hydrolysis directly on the 2 cc. urine sample to be used for the determination, carrying out both the hydrolysis and subsequent oxidation with the  $\text{H}_2\text{O}_2\text{-FeCl}_3$  in the same tube, but were unable to obtain accurate results. It was necessary to introduce this extra step to make the procedure reliable.

The total sulfur determination is the simplest to perform and can be employed with any urine, or any biological material, for that matter. 1 cc. of the original phosphate-free urine filtrate is measured into a large Pyrex tube and is digested over an electric burner with 0.5 cc. of fuming nitric acid, and 15 to 20 drops of superoxol are added from time to time. The digestion is carried to complete dryness, and, if the residue is not perfectly white, the tube is allowed to cool, the contents are taken up in a little water, and the process repeated with smaller quantities of nitric acid and superoxol. Usually, however, one digestion is sufficient to obtain the pure ash. The ash is dissolved in a small amount of water and boiled to dryness, this process being repeated twice, in order to drive off every trace of the nitric acid or peroxide. On cooling, the residue is dissolved in 5 cc. of water acidified with hydrochloric acid. Then 5 cc. of the  $\text{BaCrO}_4$  reagent are added and, after waiting 15 min-

utes, 5 cc. of the dilute ammonia. The mixture is then centrifuged as before and an aliquot portion of the clear yellow fluid is titrated with the standard thiosulfate, as usual, after adding the KI and  $\text{H}_2\text{SO}_4$ .

We have repeatedly checked the procedure by determining the sulfur in known quantities of cystine, and invariably obtained perfect results.

The factor for converting the cc. of 0.005 N (or 0.001 N)  $\text{Na}_2\text{S}_2\text{O}_3$  to corresponding sulfur values is determined by carrying out the determination as outlined, but using solutions of known sulfur content. This factor should be determined by each worker, but we found that for the inorganic or total sulfate this factor was 0.0523 mg. of S and for the total sulfur 0.0530 mg. of S, per cc. of 0.005 N

TABLE I  
*Determination of Total Sulfur in Urine*

Urine sample	S per 100 cc.		Difference
	Gravimetric	Titrimetric	
	mg.	mg.	per cent
A	98.6	98.7	+0.1
B	88.2	86.8	-0.6
C	60.5	59.5	-1.5
D	61.8	61.5	-0.5
E	134.7	132.6	-1.5
F	15.0	15.2	+1.2

$\text{Na}_2\text{S}_2\text{O}_3$ . In carrying out the determination of the factor for the total sulfur by means of known amounts of sulfate salts it is important to bear in mind that a large excess of base must be present when the digestion with the fuming  $\text{HNO}_3$  is made, as otherwise some  $\text{SO}_4$  is lost. Our urine filtrates, of course, contain such an excess of base already; but when working with small amounts of sulfate salt a few drops of concentrated KOH should be added, which insure against loss of sulfate.

We are recording in Tables I to III the results of determinations on the sulfur partitioning in human urines, which we checked either by the usual gravimetric procedure or by the Fiske benzidine precipitation (2). Frequently the sulfates were determined on the same urine by all three methods. We have also used the Denis

TABLE II  
*Sulfur Partitioning in Human Urine*

Subject	Method	S per 100 cc.		
		Total S	Total SO <sub>4</sub>	Inorganic SO <sub>4</sub>
		mg.	mg.	mg.
M.H.	Gravimetric	61.8	53.3	49.8
	Titrimetric	61.5	55.6	51.1
	Per cent difference	-0.5	+4.2	+2.5
J. M.	Gravimetric	132.7	120.1	102.3
	Titrimetric	130.3	118.8	104.6
	Per cent difference	-1.7	-1.0	+2.2
J.B.	Gravimetric	15.0	13.6	11.8
	Titrimetric	15.2	13.8	12.1
	Per cent difference	+1.2	+1.4	+2.5
"	Gravimetric	40.2	35.2	32.4
	Titrimetric	39.1	36.4	32.5
	Per cent difference	-2.7	+3.2	+0.2
T.	Gravimetric		38.9	35.5
	Titrimetric	40.5	37.0	35.4
	Per cent difference		-4.8	-0.2
"	Gravimetric		27.6	25.9
	Titrimetric	30.8	26.4	25.8
	Per cent difference		-4.2	-0.4
S.	Gravimetric		59.1	55.9
	Titrimetric	76.5	58.4	53.6
	Per cent difference		-1.2	-4.0
C.	Gravimetric	55.8	48.1	45.5
	Titrimetric	55.5	49.0	45.9
	Per cent difference	-0.4	+1.8	+0.7
H.	Gravimetric	48.9	40.1	36.0
	Titrimetric	49.4	40.0	37.0
	Per cent difference	+1.0	-0.2	+2.7
X.	Gravimetric		56.0	52.4
	Titrimetric	59.3	58.5	52.8
	Per cent difference		+4.4	+0.7

and Reed nephelometric method (3), but it failed to give reliable results in our hands. The total sulfur determination by our iodometric titration is so reliable and checks so well either with known amounts of substance or with the gravimetric procedure, that in the analyses reported in Tables II and III it was not invariably checked by the other methods.

The results by the Fiske method generally tend to be somewhat higher than by the gravimetric method, whereas the results by our iodometric procedure seem to be somewhat lower. We tested the matter by using urine made phosphate-free by our procedure for the Fiske analysis and urine prepared by Fiske's procedure for our method of analysis. The results obtained by the two methods

TABLE III  
*Comparison between Gravimetric, Benzidine, and Titrimetric Sulfur Determination*

Urine sample No.	S per 100 cc.				
	Inorganic SO <sub>4</sub>			Total SO <sub>4</sub>	
	Gravimetric	Benzidine	Chromate	Benzidine	Chromate
	mg.	mg.	mg.	mg.	mg.
1	30.7	35.7	29.7	37.6	33.7
2	36.0	38.0	34.3	42.5	41.5
3	23.7	23.8	22.7	28.2	28.9
4	30.3	31.2	29.0	34.1	32.1
5	33.5	35.6	31.4	38.3	33.7

on urines treated similarly are practically identical. However, when the results by both procedures are compared with the gravimetric determinations on the same urines the latter correspond best with our results.

#### *Determination of Total Base*

Because the determination of the total sulfur by our titrimetric procedure is very simple, rapid, and reliable, we applied it to the determination of total base. For the removal of phosphates preliminary to the total base determination of sulfates we followed the procedure recommended by Fölling (4). The material (urine, etc.) is ashed dry in a quartz dish. 4 or 5 drops of 50 per cent H<sub>2</sub>SO<sub>4</sub> are added to the material, which is then evaporated on a

water bath and ashed over an open flame. After cooling, the ash is mixed with about 1 gm. of metastannic acid<sup>1</sup> and 3 cc. of 35 per cent HNO<sub>3</sub>, the contents stirred well with a quartz rod, evaporated nearly to dryness, again taken up in 3 cc. of nitric acid, and finally evaporated to complete dryness. After cooling, 10 or 15 cc. of an alcohol-HNO<sub>3</sub> mixture<sup>2</sup> are added and well stirred. The solution is filtered through a small ashless paper and a 5 cc. aliquot (equal to one-half or one-third of the original material ashed) is evaporated in a quartz crucible, a drop of 50 per cent H<sub>2</sub>SO<sub>4</sub> being added. The crucible is heated to redness on an open flame for 10 minutes, the flame being applied so that all parts of the crucible have been heated. We have repeatedly tested the

TABLE IV  
*Total Base Determination*

Mixture of		Total base	Base found	Difference
KH <sub>2</sub> PO <sub>4</sub>	KCl			
<i>m.-eq.</i>	<i>m.-eq.</i>	<i>m.-eq.</i>	<i>m.-eq.</i>	<i>per cent</i>
	0 0569	0 0569	0 0574	+0.8
0 002	0 0569	0 0589	0 0588	-0.2
0 004	0 0569	0 0609	0 0605	-0.6
0 008	0 0569	0 0649	0 0642	-1.1
	0 0523	0 0523	0 0530	+1.1
0.002	0 0523	0.0542	0 0538	-0.9
0 004	0 0523	0 0563	0 0560	-0.5
0.006	0 0523	0 0583	0 0579	-0.7

solution of the residue for free acid, with phenol red as indicator, but have never found any trace. The ignited residue is then dissolved in 5 cc. of water acidified with HCl. This is followed by 5 cc. of the BaCrO<sub>4</sub> reagent and, after 15 minutes, by 5 cc. of the

<sup>1</sup> The metastannic acid is made by placing 20 gm. of tin shavings in 300 cc. of 35 per cent nitric acid and, when the reaction is completed, washing it several times, first by decantation, then by centrifuging. The white residue is suspended in water in a glass-stoppered jar. Just enough water is to be used to give it the consistency of a paste.

<sup>2</sup> The alcohol-HNO<sub>3</sub> mixture is prepared by diluting 2 cc. of concentrated acid to 100 cc. with water, then adding 10 cc. of alcohol. The alcohol should be added just before use. A variable quantity of the mixture can be made up, depending upon the number of analyses.

dilute ammonia. The contents are well stirred, centrifuged, and aliquots are treated with KI and  $\text{H}_2\text{SO}_4$  and titrated with 0.005 N  $\text{Na}_2\text{S}_2\text{O}_3$ , as already described. The factor for converting the cc. of thiosulfate to corresponding values of sulfur, or equivalents of base, must be determined by using known amounts of base and going through the determination in all its details. We have done so successfully, using known solutions of KCl or  $\text{K}_2\text{SO}_4$  for the standardization. We tested the procedure with known mixtures of potassium phosphate and chloride, and the results are recorded in Table IV. In our standardization the 0.005 N  $\text{Na}_2\text{S}_2\text{O}_3$  was found to be equal to 0.0520 mg. of S, or 0.00324 milli-equivalents of base, per cc.

TABLE V

*Milli-Equivalents of Total Base per Cc. of Urine as Determined by Benzidine (B.) or Chromate (Ch.) Method*

Urine volume	Urine 1		Urine 2		Urine 3		Urine 4		Urine 5	
	B.	Ch.	B.	Ch.	B.	Ch.	B.	Ch.	B.	Ch.
cc.	m.-eq.	m.-eq.	m.-eq.	m.-eq.	m.-eq.	m.-eq.	m.-eq.	m.-eq.	m.-eq.	m.-eq.
1.0	0.142	0.138	0.142	0.135	0.098	0.093	0.148	0.136	0.104	0.107
1.2							0.148	0.142		
1.4	0.138	0.128	0.133	0.129	0.100	0.094	0.144	0.142		
1.8	0.138	0.138	0.137	0.130	?	0.094	0.140	0.141		
2.0	0.137	0.131	0.135	0.130	0.099	0.096	0.148	0.137		
Average..	0.139	0.134	0.137	0.131	0.099	0.094	0.146	0.140	0.104	0.107

The results show that the analysis of total base by our iodometric titration procedure, Fölling's technique being used for removing the phosphates, is of a high degree of accuracy, and that the method is applicable to the study of very small concentrations of base.

We have also analyzed a series of urine samples, following exactly the same procedure as outlined above, and as a check we carried out the determination with variable quantities of the same urine (1.0, 1.2, 1.4, 1.8, and 2.0 cc.) to compare the results obtained within such a series. Furthermore, a parallel series of determinations was made, the Fiske benzidine method being used for determining the sulfates. The results are reported in Table V.

We have also used this method for total sulfur determination in blood and organs, but this will be taken up in a subsequent paper.

## SUMMARY

A method is described for determining sulfates by precipitation with barium chromate and titration of the equivalent amount of chromic acid set free iodometrically. The phosphates must first be removed to carry out this determination. In the absence of organic matter this procedure is quick, reliable, and suitable for the analysis of very small quantities of sulfur. For this reason the method lends itself especially well to the determination of the total sulfur content of biological material. The material, freed from phosphates by means of solid  $\text{Ca(OH)}_2$ , is digested with fuming nitric acid and superoxol. On dissolving the ash, the sulfates are determined very quickly by precipitation with  $\text{BaCrO}_4$  and by titrating the chromic acid iodometrically.

To determine the inorganic sulfates in biological material it is not sufficient, however, to remove only the phosphates, as there are other interfering substances, especially uric acid, which must be removed. This is accomplished by a mild preliminary oxidation with superoxol and a trace of  $\text{FeCl}_3$ . The method has been worked out for human urine, where the sulfur partitioning can be performed satisfactorily by our titrimetric procedure. For the total base determination the phosphates are removed according to Fölling's procedure with metastannic acid, and the dry ashed residue, dissolved in acidulated water, is treated in the same way.

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## THE EFFECT OF AN ACID EXTRACT OF THE ANTERIOR PITUITARY ON THE IODINE CONCENTRATION OF THE BLOOD AND THYROID GLAND\*

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Loeb and Bassett (1) and Loeb, Bassett, and Friedman (2) have shown that the injection of acid extracts of anterior pituitary prepared in their laboratory from cattle gland have a markedly stimulating effect upon the thyroid gland; so that the histological picture is comparable to that characteristic of the thyroid in typical Graves' disease in man. The parallelism between the intensity of hypertrophy changes and the fall in the weight curve of the guinea pigs which were used suggested to them that the administration of the extracts caused an increased elimination of thyroid hormones into the circulation and thus caused an increase in metabolism. Subsequent investigations in their laboratory by Siebert and Smith (3) have shown that this is the case; acid extracts of the pituitary glands causing a marked and rapid rise in the basal metabolism of guinea pigs. It was also shown that the increase in metabolism does not occur after complete thyroidectomy and is apparently entirely dependent upon the changes which the

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† We are indebted to Miss Hilda Friedman for her assistance in these experiments which were begun during the winter of 1930-31.

‡ The analyses in Experiments 3 and 4 were carried out by one of the authors (C.) in the Department of Pharmacology at Oslo, Norway, and he desires to thank Professor Klaus Hansen for his kindness in making this possible.

extract produces in the thyroid gland. A further analogy of the condition produced by the injections of pituitary extract with Graves' disease in man is found in Silberberg's experiments (4). He showed that the administration of KI reduced the thyroid changes ordinarily produced by the extracts.

These experiments were undertaken to study the effect of acid anterior pituitary extracts upon the iodine concentration of the thyroid and blood and to determine whether or not changes in the iodine concentration in these tissues similar to those found in Graves' disease ensued. Lunde, Closs, and Wülfert (5) have shown that the total iodine content of the thyroid gland of toxic goiters is both absolutely and relatively decreased while the total blood iodine concentration on the other hand has been found by Lunde *et al.* (6) and others (7, 8) to be definitely increased. The decrease in the thyroid iodine is in the acetone-insoluble or globulin iodine fraction (9) and the increase in the blood iodine is also in the protein fraction (6); it is therefore reasonable to suppose that there is an abnormal elimination of the thyroid hormone by the gland into the circulation in Graves' disease.

#### EXPERIMENTAL

In Experiment 1 eight guinea pigs, ranging in weight between 170 and 210 gm., were injected five times during a period of 6 days with 1 cc. of the acid extract of anterior pituitary daily. The pooled thyroid glands from these animals, which were used for analysis, weighed 384 mg. Eight blood specimens totaling 20.7 gm. in weight were analyzed separately. Thyroids weighing together 284 mg. from twelve control guinea pigs of a similar weight were pooled for analysis. Blood specimens from eight of these with body weights from 185 to 240 gm., and weighing together 24.0 gm., were analyzed separately.

In Experiment 2 the injected guinea pigs received the acid extract for 6 days instead of for 5 days. The pooled thyroids of the twelve injected animals weighed 705 mg. and the twelve controls 348 mg. Specimens of blood from six animals of the injected group totaled 18.9 gm. and from the same number of the control group, 25.7 gm. These were analyzed separately.

In Experiment 3 thyroid glands (344 mg.) were pooled from twelve control guinea pigs and individual blood specimens totaling

29.8 gm. collected from six of these for analysis. Six animals were given 1 cc. of the pituitary extract daily for 6 days. The pooled thyroids (314 mg.) and two individual and two blood specimens pooled from a couple of animals were analyzed. These totaled 25.1 gm.

The control group of Experiment 3 also served as controls for Experiment 4. Six guinea pigs received 2 cc. of the extract each day. Their thyroids (326 mg.) and the blood specimens from two pairs were pooled for the iodine determinations. The two remaining blood samples were examined individually. The blood samples totaled 19.1 gm.

The thyroid glands and the blood specimens were collected in iodine-free ethyl alcohol. The loss of alcohol from a number of blood specimens in Experiment 1 impairs the value of the figure for total iodine content in these cases but is without any effect upon the alcohol-insoluble or protein iodine values. The total and alcohol-insoluble iodine concentrations of the pooled thyroids and individual blood specimens in each experiment were determined by von Fellenberg's method (10) as modified by Lunde and Closs (11). Additional modifications which were used are described by Closs (12). The results of the blood analyses comprise Table I. The figures for the alcohol-soluble and total iodine in Experiment 1 are of doubtful significance because of the alcohol loss noted above. The alcohol-insoluble figures for the control group of Experiment 1 marked with an asterisk were obtained by the method of Veil and Sturm (7). We have reason to believe that their method gives a poor extraction of alcohol-soluble substance and consequently insoluble iodine figures which are somewhat too high. In Experiments 3 and 4 a number of determinations was lost through unavoidable technical difficulties. However, both the collection of specimens and the chemical determinations were generally satisfactory throughout.

### *Results*

The gross appearance of the thyroids from the injected guinea pigs showed that they were obviously much larger than the controls. This observation is in accordance with the gain in weight of the thyroid glands in injected animals (13). As was noted elsewhere (1, 2) the controls gained weight while the injected ani-

mals lost weight. The animals serving as controls in Experiments 3 and 4 gained an average of 25 gm. each while the injected animals in these experiments sustained an average loss in body weight of 12 gm.

The blood iodine figures are tabulated in Table I and summarized in Table II. It is true that there is considerable variation in

TABLE I  
*Blood Iodine*

Experiment No.	Control guinea pigs				Injected guinea pigs				Experiment No.	Control guinea pigs				Injected guinea pigs				
	Guinea pig No.	Total iodine	Alcohol-soluble iodine	Alcohol-insoluble iodine	Guinea pig No.	Total iodine	Alcohol-soluble iodine	Alcohol-insoluble iodine		Guinea pig No.	Total iodine	Alcohol-soluble iodine	Alcohol-insoluble iodine	Guinea pig No.	Total iodine	Alcohol-soluble iodine	Alcohol-insoluble iodine	
1	13	39	16*	23*	3	42	10	32	3	6			48	7a}	70	11	59	
	12	24	10	14	4	43	12	31		3			28	7b}				
	9	38	26*	12*	7	32	10	22		2			24	8a}	88	30	58	
	10	39	27	12	6	23	3	20		4	32	13	19	8b}	9	57	11	46
	11			10*	8	35	17	18		1	31	13	18		10	51	11	40
	15	17	7*	10*	5	21	17	4		5		19						
	14	16	7	9	1	52												
	16	12	4	8	2	44				4					12a}			
														12b}				65
	2	9	44	22	22	6	87	28	59						11a}	66	15	51
7		41	22	19	2	52	9	43						11b}	14	63	22	41
8		21	2	19	3	62	22	40							13	9		
10		16	8	8	1	55	21	34										
11		22	14	8	5	44	12	32										
12		25	21	4	4	20	12	8										

\* Determined according to Veil and Sturm (7).

the individual figures but this is usually the case with blood iodine determinations. The important result is that the findings in each experiment are essentially the same (Table II) and when all of the figures are treated as a whole they are statistically significant. The average for the nineteen control bloods is 16  $\gamma$  per cent and for the twenty-three (weighted for three pairs of pooled bloods) bloods

from injected animals 41  $\gamma$  per cent, a difference of 25  $\gamma$  per cent, which is more than 8 times the probable difference between these averages of 3  $\gamma$  per cent. It is also interesting to note how much higher are both the total and alcohol-insoluble iodine concentrations in the blood of the normal guinea pig in which a concentration of total iodine of 28  $\gamma$  per cent and in one group of even 42  $\gamma$  per cent is found than in man and the dog with blood iodine values of 14  $\gamma$  per cent (6) and 12  $\gamma$  per cent (7) respectively.

The results for the thyroids (Table III) naturally have a higher degree of accuracy than the blood iodine determinations because of

TABLE II  
*Blood Iodine Averages*

Group	Experiment No.	Total iodine	Alcohol-soluble iodine	Alcohol-insoluble iodine	Experiment No.	Total iodine	Alcohol-soluble iodine	Alcohol-insoluble iodine
		$\gamma$ per cent	$\gamma$ per cent	$\gamma$ per cent		$\gamma$ per cent	$\gamma$ per cent	$\gamma$ per cent
Control . . . . .	1	(27)		12	3	(42)	15	27
Injected . . . . .		(37)		21		66	16	54
Per cent change . . .		(+39)		+75		(+57)	0	+100
Control . . . . .	2	28	16	13	4	(42)	15	27
Injected . . . . .		53	17	36		65	15	55
Per cent change . . .		+89	0	+179		(+57)	0	+104

Parentheses indicate total iodine values which may be questionable due to alcohol loss.

the much higher concentration of iodine in the former. The significant finding is the consistent decrease in the concentration of alcohol-insoluble iodine in every experiment. The average for the control thyroids of all experiments is 28.5 mg. per cent and for the injected thyroids 7.2 mg. per cent. The actual difference between these averages, 21.3 mg. per cent, is nearly 20 times the probable difference of 1.1 mg. per cent. Although the application of the statistical method to such small groups is very questionable, such a demonstration seems hardly necessary.

There is an increase in the alcohol-soluble iodine content of the thyroid gland after the injection of the acid extract. This applies

especially to the absolute increase in alcohol-soluble iodine. It is still present although less marked if we compare the percentage increase in this iodine fraction. In view of the concordance of this result in all four experiments we wish to mention this finding, although we can at present not attribute so much importance to it as to the changes in the alcohol-insoluble iodine owing to certain variations inherent in the method.

TABLE III  
*Thyroid Glands*

Averages of groups of rats listed under each experiment in Table I.

Experiment No.	Group	Thyroid gland*	Total iodine		Alcohol-soluble iodine		Alcohol-insoluble iodine	
		mg.	mg. per cent	mg. per gland	mg. per cent	mg. per gland	mg. per cent	mg. per gland
1	Control	23.6	30.2	7.2	1.0	0.2	29.2	7.0
	Injected	48.0	12.4	6.0	1.2	0.6	11.2	5.4
	Per cent change	+104	-59	-17			-62	-23
2	Control	29.0	33.0	9.6	1.6	0.5	31.4	9.1
	Injected	58.7	9.2	5.4	4.4	2.6	4.8	2.8
	Per cent change	+102	-75	-44			-85	-69
3	Control	28.7	27.4	7.9	0.6	0.2	26.8	7.7
	Injected	50.7	6.5	3.3	1.1	0.5	5.4	2.8
	Per cent change	+80	-79	-58			-80	-63
4	Control	28.7	27.4	7.9	0.6	0.2	26.8	7.7
	Injected	54.3	15.2	8.2	7.8	4.2	7.4	4.0
	Per cent change	+90	-45	+3			-73	-48

\* Average weight per rat.

#### DISCUSSION

An examination of the data presented in Tables I to III shows very clearly that the injection of the acid anterior pituitary extract caused an increase in the organic blood iodine at the expense of the organic iodine content of the thyroid gland. The important thyroid iodine figure is naturally the concentration in the gland. Not only is this definitely reduced but the amount of iodine per gland is also significantly less even though the glands of the injected animals are twice the weight of the controls. These find-

#### CORRECTIONS

On page 590, Vol. xcvi, No. 3, June, 1932, Table III, Columns 5, 7, and 9, read *γ per gland* for *mg. per gland*.

ings harmonize well with the histological changes which result from the injections of acid extracts. As Loeb and Bassett (1) and Loeb *et al.* (2) have shown, the hypertrophy of the thyroid gland produced under these conditions is associated with a marked decrease in the amount of colloid in the acini, and what is left of it stains very poorly with eosin, a condition pointing to a marked liquefaction and absorption of the colloid. Inasmuch as the iodine in the thyroid is mainly contained in the colloid, the fact that by far the greater part of the colloid in the individual acini has been liquefied and absorbed justifies the assumption that a diminution in the amount of iodine material has taken place in the thyroid, not withstanding the marked proliferation of the acinar epithelium and the resulting increase in the amount of gland tissue which has taken place as a result of the administration of anterior pituitary extract. With these results harmonize in some respects the results recently obtained by Loeser (14) who found that injection of anterior pituitary in the dog causes an almost total loss of the iodine in the thyroid gland. This author did not distinguish between the alcohol-soluble and alcohol-insoluble iodine fraction in the thyroid gland.

The experiments reported here strongly suggest the conclusion that the injection of anterior pituitary extract not only causes the thyroid hypertrophy, the changes in glandular histology, and the increased basal metabolic rate, but also reproduces in the organism of the guinea pig the changes in organic iodine distribution which are characteristic of Graves' disease in man.

#### SUMMARY

By means of injections of acid extracts from anterior pituitary of cattle, changes in the iodine metabolism (the concentration of alcohol-insoluble or "organic" iodine increasing in the blood and decreasing in the thyroid) can be produced in the guinea pig which correspond to those noted in Graves' disease in man.

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## FACTORS IN FOOD INFLUENCING HEMOGLOBIN REGENERATION

### I. WHOLE WHEAT FLOUR, WHITE FLOUR, PREPARED BRAN, AND OATMEAL\*

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The technique of inducing anemia in rats by milk feeding is now well understood, and interest centers in the substances which are effective in the cure of this nutritional disorder. Reports are conflicting with regard to the rôle of various inorganic elements, but there is no dissent from the view that iron is essential and can be utilized when added to the diet in the form of soluble inorganic salts. That regeneration can take place with addition of iron only is denied by most workers from Bunge (1889) to the present time. Hart, Steenbock, Waddell, and Elvehjem (1) were the first to show clearly the marked influence of copper in hemoglobin regeneration in nutritional anemia, and this effect has been confirmed by every subsequent worker in the field.<sup>1</sup> According to Mitchell and Miller (6) the smallest amount of iron which will induce normal regeneration in a milk-fed anemic rat is 0.25 mg. per day. What additional stimulation may be derived from larger doses of iron is not yet clear. There is some evidence that with considerable copper in the milk ingested (and probably some in reserve in the rat body) the dosage of iron may be so increased as to secure good results without adding more copper to the diet (3). This is in line with clinical results from iron administration in various types of secondary anemia as recently reported by Minot and Heath (7), but is contrary to the

\* Based on a thesis presented by E. McC. Vahlteich in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

<sup>1</sup> For reviews of the literature see Smith (2), Beard and Myers (3), Cunningham (4), and Rose (5).

findings of Waddell, Elvehjem, Steenbock, and Hart (8), who fed 0.5 mg. of iron six times per week as ferric chloride, acetate, citrate, phosphate, or sulfate with negative results in every case. The copper content of the milk fed by Elvehjem, Steenbock, and Hart, (9) was, however, lower than that reported by any other investigator, so that copper rather than iron may have been the limiting factor in their animals.

According to Myers and Beard (10) a whole category of other mineral elements exerts some supplementary effect, but none has been tried in complete absence of copper from the daily food intake and none has been found effective without more iron than Mitchell and Miller (6) find necessary to induce normal regeneration when supplementary copper is furnished. That there may be such supplementary effects seems quite likely. Whipple and Robschey-Robbins (11) have repeatedly called attention to a "salt effect" in the anemia of hemorrhage. The experiments of Titus, Cave, and Hughes (12), who found manganese an effective supplement, and of Myers and Beard (10), who claim stimulating effects with so many different metals (including zinc which has been reported as inert by all others who have tested it), await further elucidation.

Some evidence that organic compounds may also play a part in the cure of nutritional anemia has been brought forth by Drabkin and Miller (13) who found that administration to rats, made anemic by milk feeding, of pure arginine monohydrochloride in doses of 100 mg. plus 0.2 mg. of iron daily resulted in a hemoglobin increase, although there was not enough copper in 0.5 gm. of sample to be determined quantitatively. Glutamic acid, arginine, and tryptophane also induced good response, but alanine,  $\alpha$ -aminovaleric acid, aspartic acid (as sodium hydrogen aspartate), and pyrrolidonecarboxylic acid were ineffective. Proline, succinic acid, and succinimide were only slightly stimulating. How far these effects are related to the amount of iron administered, it is difficult to determine. With 0.18 mg. of iron per rat per day added to the diet, increasing amounts of glutamic acid up to 70 mg. gave increasingly good regeneration. But with less iron or none at all added and a constant dosage of glutamic acid (70 mg.) the amount of regeneration seemed to be dependent upon the amount of iron administered. These results could not be confirmed by Elvehjem, Steenbock, and Hart (14), who were unable to secure any regenera-

tion with 0.5 mg. of pure iron plus 100 mg. of glutamic acid hydrochloride. This again is contrary to the report of Beard and Myers (3) who obtained good regeneration with 0.5 mg. of iron alone. The chief difference appears to be in the amount of copper ingested, the latter workers reporting a copper content of 0.44 mg. per liter for their milk, while the Wisconsin investigators used milk yielding only about 0.15 mg. of copper per liter. It is quite evident that no satisfactory conclusion as to the relative importance of iron and copper or of any other factor in the cure of nutritional anemia can be reached without fuller knowledge of the food intake of the animals. So far, the only synthetic diets which have been employed are those of McCay (15), Drabkin and Waggoner (16), and Cunningham (4).

The present investigation was begun in 1928 (17) when Whipple and Robscheit-Robbins (11) were stressing the marked differences in the regenerating power of various natural foods in hemorrhagic anemia and when our own studies of the place of cereals, eggs, and vegetables in children's diets (18, 19) gave us special interest in the value of whole wheat as a source of iron in children's diets. At first in attempting to find a basal diet more satisfactory than fresh cow's milk, we tried substitution of white flour for part of the milk, but found, to our surprise, that it was impossible to induce anemia unless the animals were restricted to milk exclusively.

The only other study of any cereal food so far reported is one by Waddell, Elvehjem, Steenbock, and Hart (8) who observed no response from corn or wheat when fed to rats in doses of 2 gm. of cereal (containing 0.15 mg. of iron) six times per week. Cereals are an important staple in the diet and are capable of furnishing a considerable proportion of the daily iron requirement of human beings at very low cost, provided the iron can be efficiently absorbed and utilized. Sherman (20) in his classic study of 224 American dietaries found that grain products which furnished 38 per cent of the total calories also furnished about 25 per cent of the total iron intake, and Rose and Gray (18) in their study of dietaries in children's institutions found cereal grains contributing in one institution about 18 per cent of the total iron ingested and in two others from 30 to 38 per cent of the total iron. In a fourth, white bread and milk furnished over half the total iron, and tests of 73 children who had subsisted on this diet a year or more showed

normal hemoglobin values. Cereals furnish in addition to iron a variety of other mineral elements, among which copper is at the present time of special interest as a factor in iron utilization for hemoglobin building. According to Cunningham (4) it is concerned with the mobilization of iron from the liver and is stored in the liver before birth to serve as a reserve during lactation, just as Bunge showed iron to be stored.

Up to the present time very few studies of the relative influence on nutritional anemia of the various mineral elements present in natural foods as compared with the foods themselves have been reported. Mitchell and Miller (21) tried a water extract of spinach and found that quantities furnishing respectively 0.5 mg. and 0.25 mg. of iron six times per week were almost equally effective. The ash of this extract did not bring quite so rapid a response, but the hydrochloric acid solution of the ash furnishing 0.5 mg. of iron was as effective as the original extract. Supplementing the milk with 0.5 mg. of iron plus 0.05 mg. of copper alone or with 0.05 mg. of manganese resulted in slower regeneration than use of the extract or of the ash, although both contained 0.014 mg. of copper and 0.012 mg. of manganese per 0.5 mg. of iron.

In the present study data of a quantitative nature are offered to show the value, for hemoglobin regeneration of rats made anemic by an exclusive milk diet, of wheat in the forms of whole wheat flour, white flour, and prepared wheat bran. Tests were made on the ash of whole wheat and of bran, and the white flour was supplemented with copper or iron or both. Some data on oatmeal are also included for the purpose of comparison.

Young rats 28 to 30 days of age, raised on Sherman's Diet 13 ( $\frac{2}{3}$  whole wheat,  $\frac{1}{3}$  milk powder, sodium chloride equal to 2 per cent of the weight of the wheat) with fresh lean beef and lettuce as supplements two or three times a week became distinctly anemic in 5 to 7 weeks on a diet of milk only. At first pasteurized milk from a large city market was used but the production of anemia was slow and irregular, hence to avoid contamination with copper, certified city milk was substituted. In preliminary tests it had been found that anemia resulted equally soon whether glass or galvanized iron cages with  $\frac{3}{8}$  inch mesh wire for the bottoms were used. With  $\frac{1}{4}$  inch mesh wire the consumption of feces kept the animals from becoming anemic, consequently new galvanized iron cages with  $\frac{3}{8}$  inch mesh

for the bottoms were adopted for this work. This experience regarding cages is in harmony with the findings of Beard and Myers (3). When the hemoglobin fell to about half normal (preferably 5 to 7 gm. per 100 cc. of blood), each rat was placed in a separate cage and the experimental period was started. The first thirty-seven animals (twenty-five fed milk plus supplements, twelve fed milk only) were observed through a period of 11 weeks, but thereafter the experimental period was shortened to 6 weeks, since complete regeneration was found to take place in that time. —

Mitchell and Miller (6) have called attention to the fact that it is important to obtain uniformly low hemoglobin values for all animals before starting the experimental period, in order to obtain consistent results, because their animals with higher initial hemoglobin values responded faster than those with lower ones. A low and uniform hemoglobin level is undoubtedly a very important detail of technique and one which we have endeavored to adhere to, but it may be noted in passing that in our experience animals with very low initial hemoglobin values may or may not respond better than those with somewhat higher initial values; *e.g.*, on 6 gm. of whole wheat, two animals, depleted to 4.3 and 4.7 gm. of hemoglobin, regenerated in 6 weeks 9.8 and 11.1 gm. respectively. Similar results were observed in each series and among animals of the same age.

After the 3rd week of the depletion period all hemoglobin determinations were made weekly. The Newcomer method (22) with the same Newcomer disk was used throughout. The blood was procured by snipping off a small amount of the end of the rat's tail. Free flowing drops were always used and duplicate samples were taken at each bleeding. Copper and iron determinations were made on the supplements (Table I). The Biazzo method as modified by Elvehjem and Lindow (23) was used for copper and the Zimmermann-Reinhardt as modified in this laboratory (24) for iron. The certified milk was analyzed at two different periods for iron (three different samples, April, 1929, and three others in March, 1931). The results varied from 0.5 to 1.0 mg. per liter, averaging in both years 0.8 mg. Elvehjem (25) has pointed out the danger that any method involving ashing at high temperature in presence of large amounts of phosphates tends to give low returns for iron, but we found that hydrolysis of the ash by boiling for 1

hour with sodium hydroxide did not alter the values of iron in our samples. Stugart (26) using a method with which he maintains that he obtains reliable results in presence of pyrophosphates, has reported for certified milk values from 0.46 to 0.57 mg. of iron per liter. An analysis of the milk for copper, made in January, 1932, showed a content of 0.27 mg. of copper per liter.

The solutions of ferric chloride and copper sulfate for feeding were each made to such a volume that 1 cc. of aqueous solution would contain the daily dosage. Two different samples of one commercial brand of ground whole wheat were used, known to be made from the whole grain; two lots of one brand of patent flour and one lot of prepared bran were used as purchased. Three lots

TABLE I  
*Iron and Copper Content of Supplements*

	Sample No.	Iron	Copper
		<i>mg. per 100 gm.</i>	<i>mg. per 100 gm.</i>
Whole wheat (air-dry)	I	0 34	0.071
	II	0 32	
Oatmeal "	I	0.36	0.090
	II	0 32	0 078
	III	0 31	0 085
Patent flour "	I	0 05	0.022
	II	0 05	0 026
Prepared bran "		1 10	0.068
Ferric chloride			0.150

of one brand of rolled oats were used, these being ground to a meal in the laboratory in a carefully cleaned grinder with highly polished steel burrs. For convenience the supplements were fed three or four times weekly, but they will be referred to in terms of the average daily dosages, based on a 7 day week, so that they may be more easily compared with those of other investigators.

*Food Consumption and Growth*—The food consumption of each animal as recorded daily was calculated to calories per gm. of rat per day and to percentage calories from the food supplements. The figures for calories per gm. of rat per day were very uniform (0.22 to 0.31) and are within the same range as those reported by Campbell (27) for rats of similar ages on an adequate diet ( $\frac{1}{3}$  whole

#### CORRECTION

On page 598, Vol. xcvi, No. 3, June, 1932, Table I, Columns 3 and 4, read 10 gm. for 100 gm.

milk powder,  $\frac{2}{3}$  whole wheat, salt). Although the calories from milk varied with the different supplements there was seldom a lower proportion of milk than is found in Sherman's Diet 13 (see p. 596) which has been found adequate for growth. The lowest proportion of milk was ingested when 6 gm. of oatmeal were fed as a supplement, the cereal contributing on the average 61 per cent of the total calories. A few animals did not consume all of this large cereal supplement readily, and occasionally, to make them eat it, the milk was restricted on certain days. Such differences in milk intake did not appear to affect the hemoglobin production; *e. g.*, two rats consuming 59.7 and 82.2 per cent respectively of their calories in the form of oatmeal had the same hemoglobin increase in 6 weeks. Likewise animals on the same supplement, consuming the same amount of milk, sometimes showed considerable difference in hemoglobin increase; *e. g.*, two animals of about the same body weight on milk plus 1.5 gm. of whole wheat showed hemoglobin increases in 6 weeks of 7.8 and 3.4 gm. respectively.

There was some growth as a rule during the depletion period, but the rate became progressively slower as the anemia intensified. Upon the addition of the supplements, excepting white flour alone or with additional copper and also 1.5 gm. of whole wheat, there was usually some acceleration of the growth rate, proportionately better as the amount of iron was greater. There was little difference between the growth rate on the amounts of whole wheat, oatmeal, or bran carrying 0.2 mg. of iron or on the corresponding ash, although the proportion of milk varied from 100 per cent of the total calories with the ash supplements and 94 to 97 per cent with the bran to about 60 per cent with the high wheat and oats supplements.

*Effect of Whole Wheat Flour*—Whole wheat flour was fed at three levels, namely 1.5, 3, and 6 gm. a day, to lots of ten or eleven animals each. The average increases in hemoglobin in 6 weeks corresponding to the above levels of intake were respectively 4.2, 6.7, and 10.8 gm. per 100 cc. of blood. Complete regeneration took place in 6 weeks on a daily dosage of 6 gm. of whole wheat flour yielding 0.192 mg. of iron and 0.043 mg. of copper, and good regeneration took place on half that amount. A comparison of the difference between regeneration at levels of 1.5 gm. and 3 gm. of whole wheat intake, made on the basis of average figures for hemo-



globin increases for a 6 weeks period, shows that increments are roughly proportional to the amounts fed. Thus those animals on 1.5 gm. of whole wheat had an average increase of 4.2 gm. of hemoglobin and those on 3 gm. an average increase of 6.7 gm. Results in a similar comparison of 3 gm. with 6 gm. of whole wheat show a like relationship, the increase on 3 gm. being 6.7 gm. of hemo-

findings of Waddell, Elvehjem, Steenbock, and Hart (8). In the case of eight animals on 1.5 gm. we have found increases ranging from 3.4 to 7.8 gm. of hemoglobin and only two cases as low as 1.4 and 1.5 gm., whereas in case of the negative controls receiving milk alone, only one out of twenty-nine animals made a gain of 1.4 gm. Two other controls showed increases of 0.2 gm. and one of 0.8

TABLE II—Individual Increases in Gm. of Hemoglobin per 100 Cc. of Blood after Feeding Daily Supplements for 6 Weeks Experimental Period

	Whole wheat flour				Patent flour				Prepared bran			Oatmeal					Controls receiving milk alone	
	1.5 gm.	3 gm.	6 gm.	HCl solution of ash of 6 gm.	3 gm.	6 gm.	3 gm. plus <sup>a</sup>			0.9 gm.	1.8 gm.	HCl solution of ash of 1.8 gm.	1.5 gm.	3 gm.	6 gm.	3 gm. plus		
							Cu	Fe	Cu + Fe							Cu		Fe
Fe in supplement, mg.....	0.048	0.096	0.192	0.192	0.015	0.030	0.015	0.192	0.192	0.009	0.198	0.198	0.048	0.096	0.192	0.096	0.192	
Cu "																		

globin, while on 6 gm. of wheat it was 10.8 gm. These results are shown in detail in Table II and Fig. 1, A.<sup>2</sup>

The increase on 1.5 gm. of whole wheat is not in accord with the

<sup>2</sup> For each animal the difference between the lowest level of hemoglobin reached on milk and the highest level attained in 6 weeks of feeding any of the supplements has been taken as the gain for the period.

gm.; the remainder ranged from 0.0 to -4.8 gm., the total control group (Table II) averaging -1.6 gm. Therefore it is evident that our increases resulting from feeding the wheat were not due to spontaneous recovery.

Since whole wheat gave such excellent results in recovery from nutritional anemia, attention was turned toward a possible expla-

nation. Wheat, during the process of refining, loses about 75 per cent of its total ash, 8 per cent of its protein, 50 per cent of its fat, and practically all its fiber. It seemed desirable to take patent flour and add mineral supplements to see whether increases in hemoglobin similar to those made by rats fed 6 gm. daily of whole

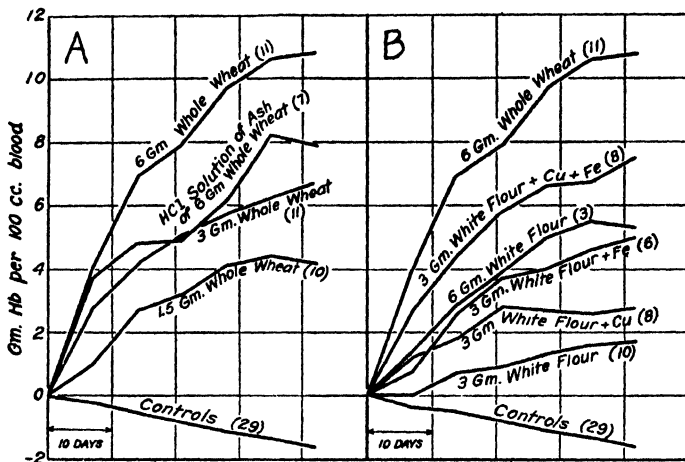


FIG. 1, A. Average weekly increases in hemoglobin from feeding daily 6 gm. of whole wheat yielding 0.2 mg. of Fe and 0.043 mg. of Cu, ash as hydrochloric acid solution of 6 gm. of whole wheat yielding 0.2 mg. of Fe and 0.043 mg. of Cu, 3 gm. of whole wheat yielding 0.1 mg. of Fe and 0.027 mg. of Cu, and 1.5 gm. of whole wheat yielding 0.05 mg. of Fe and 0.013 mg. of Cu.

FIG. 1, B. Average weekly increases in hemoglobin from feeding daily 6 gm. of whole wheat yielding 0.2 mg. of Fe and 0.043 mg. of Cu, 3 gm. of white flour + Cu + Fe yielding 0.2 mg. of Fe and 0.043 mg. of Cu, 6 gm. of white flour yielding 0.03 mg. of Fe and 0.014 mg. of Cu, 3 gm. of white flour + Fe yielding 0.2 mg. of Fe and 0.007 mg. of Cu, 3 gm. of white flour + Cu yielding 0.015 mg. of Fe and 0.043 mg. of Cu, and 3 gm. of white flour yielding 0.015 mg. of Fe and 0.007 mg. of Cu.

The figures in parentheses indicate the number of animals used.

wheat could be obtained. Since levels of 3 and 6 gm. of whole wheat had given increases roughly proportional to the amounts fed, the same amounts of white roller process flour were used. A group of ten animals each fed daily 3 gm. of white flour gave an average increase of 1.6 gm. in 6 weeks, whereas those fed 3 gm. of whole wheat gave an average increase of 6.7 gm. With larger amounts there was difficulty in getting the flour all eaten. Out of six ani-

mals fed 6 gm., there were only three which completely consumed their portions. These three gave an average increase of 5.4 gm. of hemoglobin in 6 weeks, whereas 6 gm. of whole wheat gave an average increase of 10.8 gm. It thus is quite evident that when wheat is refined the white flour still possesses some antianemic power (Table II and Fig. 1, B).

*Effect of White Flour with Mineral Supplements*—Since 85 per cent of the iron and 66 per cent of the copper are removed from whole wheat in producing patent flour, it seemed desirable to find out whether the addition of iron or copper or both as inorganic salts to white flour in amounts to make the total in each case equivalent to the quantity of each element in 6 gm. of whole wheat would bring about the same increases in hemoglobin. Accordingly, 3 gm. of white flour were supplemented (a) with 0.177 mg. of iron as ferric chloride, (b) with 0.036 mg. of copper as copper sulfate, and (c) with like amounts of both iron and copper, so that the total amount of iron was 0.192 mg. and of copper 0.043 mg., the amounts of each in 6 gm. of whole wheat. It will be seen in Table II and Fig. 1, B that neither of these elements singly nor the two in combination as supplements to white flour gave results equal to those with 6 gm. of whole wheat. Addition of iron alone raised the hemoglobin level in 6 weeks on the average 3.4 gm., while the addition of iron plus copper increased it 5.9 gm. In other words, addition of iron to a diet of white flour and milk stimulated hemoglobin production to some degree, but not as much as addition of iron and copper together. Nevertheless, the effect of the two metals together fell short of the level obtained upon feeding 6 gm. of whole wheat; likewise 3 gm. of white flour plus iron and copper to equal 6 gm. of whole wheat made a supplement little if any more effective than 3 gm. of whole wheat alone. Feeding the 3 gm. of whole wheat caused a gain of 6.7 gm. of hemoglobin and feeding the 3 gm. of supplemented white flour induced a gain of only 7.5 gm. It is evident, therefore, that the quantities of iron and copper fed are not the only factors responsible for the hemoglobin regeneration secured with whole wheat.

In these experiments in which white flour was supplemented with iron and copper, the iron was less than one-half of that used by Hart, Steenbock, Waddell, and Elvehjem (1) in studying the effect of supplementing milk with pure iron while the copper was about

the same. The increases in hemoglobin which they secured compare favorably with the increases given by 6 gm. of whole wheat, and are greater than those given by patent flour plus our ferric chloride and copper sulfate supplements. Our results from feeding 1.344 mg.<sup>3</sup> of iron per week as whole wheat were quite similar to those of Hart and his associates from feeding weekly 3.0 mg.<sup>4</sup> of iron as ferric chloride. Poorer regeneration resulted from feeding the iron partly as white flour and partly as ferric chloride, hence it would seem that the iron in whole wheat was better utilized, or that there was present some other factor or factors which served the same purpose as the extra amount of inorganic iron fed by Hart and his associates.

Since it had been found that addition of copper and iron to white flour to make 3 gm. in respect to these elements equal to 6 gm. of whole wheat did not induce the same hemoglobin production as the natural whole wheat, the hydrochloric acid solution of the ash of 6 gm. of wheat was next added to the milk ration to see whether the active agents were wholly inorganic. In one case the hemoglobin increase was 10.9 gm., and in another 9.9 gm.; but the remaining five animals ranged from 7.2 gm. down to 6.4 gm. with an average for the seven animals of 7.8 gm., which is 28 per cent less than that for 6 gm. of whole wheat (Table II and Fig. 1, A). This would indicate that, although the ash contained certain factors which, when combined with milk, were beneficial in regenerating hemoglobin, it was still not as efficient as the original wheat. If the factors involved were wholly inorganic it would seem as though equally good regeneration should have resulted, but other investigators with the exception of Drabkin and Miller (13) think only inorganic factors are involved.

*Effect of Prepared Bran*—Since prepared bran is used quite commonly as a breakfast cereal the question of the absorption of iron from a wheat product so different in physical character from whole wheat or patent white flour seemed worth investigating. Accordingly a product which is cooked and crumbled was purchased in the open market, and this was also tested for its anti-anemic properties. Two groups of twelve animals each were fed at two levels of intake, 0.9 and 1.8 gm. daily, the respective equiva-

<sup>3</sup> 0.192 mg. daily for 7 days.

<sup>4</sup> 0.5 mg. daily for 6 days.

lents in iron of 3 and 6 gm. of whole wheat. The average increases in hemoglobin were 6.0 and 9.9 gm. respectively, values very similar to those brought about by feeding portions of whole wheat containing corresponding amounts of iron (Table II and Fig. 2, A). As in case of whole wheat, the ash of the prepared bran was fed as the sole supplement to milk to nine animals with a resulting average increase in hemoglobin in the 6 weeks period of 8.2 gm. This

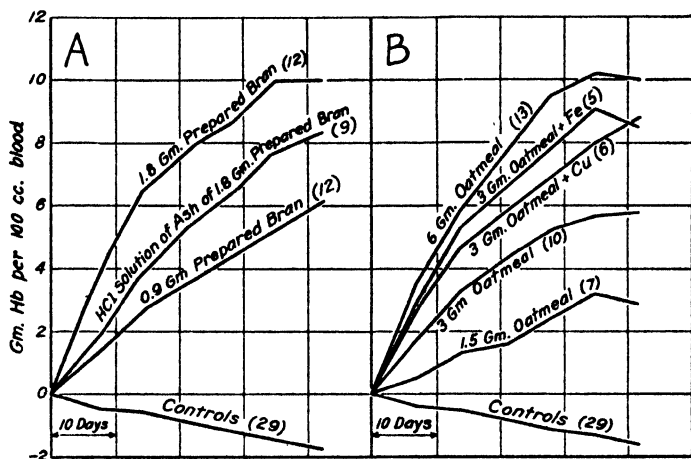


FIG. 2, A. Average weekly increases in hemoglobin from feeding daily 1.8 gm. of prepared bran yielding 0.2 mg. of Fe, ash as hydrochloric acid solution of 1.8 gm. of prepared bran yielding 0.2 mg. of Fe, 0.9 gm. of prepared bran yielding 0.1 mg. of Fe.

FIG. 2, B. Average weekly increases in hemoglobin from feeding daily 6 gm. of oatmeal yielding 0.2 mg. of Fe and 0.051 mg. of Cu, 3 gm. of oatmeal + Fe yielding 0.2 mg. of Fe and 0.025 mg. of Cu, 3 gm. of oatmeal + Cu yielding 0.1 mg. of Fe and 0.051 mg. of Cu, 3 gm. of oatmeal yielding 0.1 mg. of Fe and 0.025 mg. of Cu, 1.5 gm. of oatmeal yielding 0.05 mg. of Fe and 0.013 mg. of Cu.

The figures in parentheses indicate the number of animals used.

again, is lower than the average amount regenerated on the original bran. Not only are the averages for the group with the ash supplements lower than when the bran was fed, but the returns for the individual animals are also consistently lower, only two of the nine animals on the ash of bran reaching an increase of as much as 9 gm. while nine out of twelve on the original product reached values ranging from 9.4 to 12.2 gm.

*Effect of Oatmeal*—Oatmeal, as another staple in the American dietary which contains significant amounts of iron similarly held in the outer coats of the grain, was chosen for comparison with wheat and was fed at the same levels; viz., 1.5, 3, and 6 gm. to groups of 7, 10, and 13 animals, respectively. Again the smallest increase in hemoglobin was 2.9 gm. on 1.5 gm. of oatmeal; the highest was 10 gm. on 6 gm. of oatmeal, with the intermediate value of 5.8 gm. for 3 gm. of oatmeal (Table II and Fig. 2, B). It will be noted that the relationship between the amount of iron ingested and the increment of hemoglobin is similar to that found in the case of whole wheat and prepared bran. Whole wheat and oatmeal contain about the same percentages of copper, and when supplied in amounts equivalent as to iron are about equally good sources of the factors needed in cure of anemia induced by milk feeding.

Since iron and copper added to patent flour did not bring about the response which resulted from feeding equivalent amounts of these elements in the form of whole wheat, it was considered worth while to supplement oatmeal with iron and copper in similar fashion. Accordingly, 3 gm. were supplemented with 0.096 mg. of iron as ferric chloride and with 0.023 mg. of copper as copper sulfate, so that the total amounts were equal to those found in 6 gm. of oatmeal. The average increases in hemoglobin for two groups of five and six animals respectively were 8.5 gm. and 8.8 gm. as against 10.0 gm. for those fed 6 gm. of oatmeal. In each case the increases were greater than when 3 gm. of white flour were supplemented with both iron and copper so as to equal 6 gm. of whole wheat. Thus the oatmeal appears to have certain factors, lacking in patent flour, which supplement the inorganic iron and copper; or the iron and copper as they occur naturally in the 3 gm. of oatmeal may have been better utilized than were the inorganic iron and copper added to the white flour. The results are given in Table II and Fig. 2, B.

Since it is difficult to collect a very large number of cases in studies involving such long feeding periods and frequent hemoglobin determinations, it has been impossible to test the reliability of these results in the ordinary statistical manner. But the method suggested by "Student" (28) as extended by Fisher (29) has been applied to the data, with results which indicate that the conclusions which follow may be regarded as significant.

## SUMMARY AND CONCLUSIONS

Whole wheat and oatmeal as supplements to milk were fed to anemic rats in such quantities (1.5, 3, and 6 gm.) as to yield respectively 0.048, 0.096, and 0.192 mg. of iron daily and were found to regenerate hemoglobin in proportion to the amount given, a return to the normal level occurring in 6 weeks with 6 gm. of either cereal. Prepared bran was fed at levels of 0.9 and 1.8 gm., yielding 0.1 and 0.2 mg. of iron, respectively, and showed regeneration roughly proportional to the amount of iron. Patent flour fostered hemoglobin regeneration to some extent, better results being obtained with 6 gm. than with 3 gm. When 3-gm. of white flour were supplemented with copper, with iron, or with copper plus iron, as solutions of ferric chloride and copper sulfate to equal the amounts found in 6 gm. of whole wheat, regeneration did not in any case equal that with 6 gm. of the original whole wheat, and it was not greater than with 3 gm. of whole wheat. The addition of iron alone gave better results than addition of copper alone and addition of copper plus iron caused more improvement than addition of iron alone. 3 gm. of oatmeal to which (a) copper and (b) iron were added in amounts equaling the quantities of these elements found in 6 gm. of oatmeal induced almost if not as good regeneration as the latter. Hence the factors other than copper and iron which may influence hemoglobin regeneration are probably present in greater amounts in oatmeal than in white flour.

It would seem, then, that the cure of nutritional anemia induced in rats by milk feeding may be accomplished in 6 weeks by supplementing milk with whole wheat, prepared bran, or oatmeal in quantities to furnish as much as 0.2 mg. of iron daily, that copper as well as iron plays a part, and that a hydrochloric acid solution of whole wheat or of bran ash does not give quite as good regeneration as the original food material.

The average hemoglobin increase with the ash of 6 gm. of whole wheat was 7.8 gm. per 100 cc. of blood, about the same as with iron and copper supplements to white flour, while that of the original wheat was about 30 per cent higher. The difference between the hemoglobin regeneration induced by the ash of 1.8 gm. of prepared bran and the corresponding quantity of the bran itself was less striking but in the same direction.

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## THE DETERMINATION OF ZINC IN BIOLOGICAL MATERIALS\*

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The occurrence of zinc in biological materials is no longer a matter of conjecture. The presence of zinc in all plants and lower animals so far studied, as well as its occurrence not only in the whole body of the higher animals but in the individual tissues such as liver, kidney, muscle, etc., has been established beyond doubt. In 1926, Lutz (1) reviewed very completely our knowledge concerning the occurrence and distribution of this element in biological materials. A study of the figures presented in this review reveals that the great discrepancies in the values obtained by different workers for like materials can only be partially accounted for by variations in the zinc content of the samples; the greater part of the differences must be due to the lack of standard methods of analysis.

Workers have suggested various functions of zinc in the metabolism of plants and animals. A review by Drinker and Collier (2), however, shows that no specific activity has as yet been ascribed to this element. The lack of available methods for the accurate estimation of zinc seems to be the limiting factor in the continuation of this work.

In our attempts to utilize certain of the published methods for the estimation of zinc in plant and animal tissues, considerable difficulty was encountered. The methods which gave fairly accurate results in our hands were exceedingly time-consuming. With other methods we were unable to obtain consistent values. Attempts to modify parts of various methods in order to adapt

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them to our conditions led to the development of a new micro method for zinc. Certain of the principles involved in this method are new, while some of the reactions are based on hitherto well established procedures. By the use of this new technique we are able to complete a determination within 8 hours after ashing and limit the variations in duplicate determinations to less than 5 per cent.

The determination of zinc in biological materials requires two main steps; namely, the separation of zinc from other elements, and the quantitative estimation of the zinc so separated. A large number of methods have been used for the separation of zinc, but the majority of them depends on the precipitation of zinc as the sulfide in solutions of definite hydrogen ion concentration. Gravimetric, volumetric, turbidimetric, and colorimetric methods have been used for the quantitative determination after the zinc has been freed from other elements.

McHargue (3) and Delezenne (4) used the gravimetric method, weighing zinc as zinc sulfate. Weitzel (5) weighed zinc as the oxide, and Bertrand (6) precipitated calcium zincate and weighed this compound. It is difficult to adapt these methods to minute amounts of zinc.

Hubbell and Mendel (7) titrated zinc with potassium ferrocyanide, using uranium nitrate as outside indicator. However, the method could not be used when the samples contained less than 0.5 mg. of zinc. With smaller quantities than this the end-point in the titration was determined by the potentiometric method of Van Name and Fenwick (8).

Among the turbidimetric methods is that of Breyer (9) who made use of the turbidity obtained with potassium ferrocyanide in pure zinc solution. Bodansky (10) used a similar method and this procedure has recently been modified by Fairhall and Richardson (11). Meldrum (12) employed ammonium sulfide in a nephelometric method.

Campo and Puente (13) and Brenner (14) made use of colorimetric methods. The former workers employed resorcinol in an ammoniacal solution, and the latter investigator made use of lakes. In general these procedures have not proved successful. Undoubtedly the best colorimetric method is that of Lutz (15) who used the fluorescence produced by urobilin in an ammoniacal

alcoholic solution of the metal. Great accuracy is claimed for the method, and it is said to be adaptable to quantities as small as 0.01 mg. of zinc. We have found this procedure very long and time-consuming, and have experienced considerable trouble in attempting to obtain consistent results when different preparations of urobilin were applied to standard solutions of zinc.

The method which we wish to describe in this paper is based on the precipitation of zinc as zinc ammonium phosphate after it has been freed from the other elements present in the ash solution. The procedure used for the separation of zinc is in effect a modification of that described by Fairhall and Richardson (11) which involves the co-precipitation of zinc with added copper as sulfides at a definite pH to eliminate the bulk of the impurities, a second precipitation under identical conditions to remove any remaining impurities, and a final separation of the copper and zinc. Their procedure has been modified in an effort to simplify and shorten each step involved. For example, the sulfide precipitates are separated by centrifugation rather than by filtration after long standing. Further, the oxidation of the sulfides is accomplished by hydrogen peroxide in HCl solution rather than by nitric and hydrochloric acids. This change eliminates the need for several evaporations to insure the complete removal of nitric acid.

After the zinc is obtained in pure solution, the problem centers itself around the estimation of this element. Upon the suggestion of Dr. Loren C. Hurd of the Chemistry Department, to whom we express our thanks, we undertook the standardization of the quantitative precipitation of zinc ammonium phosphate from solutions containing minute quantities of this element. When diammonium hydrogen phosphate is used as the precipitating reagent, the zinc ammonium phosphate comes down first as an amorphous compound which upon standing changes into a crystalline salt of the composition  $\text{ZnNH}_4\text{PO}_4$ . At definite pH values and definite concentrations it was found that the zinc could be carried down with practically no loss.

This precipitate might be weighed as such or ignited to the pyrophosphate for weighing, but we chose to use other means of ultimate estimation of the zinc, thus eliminating on the one hand the necessity of a micro balance, and on the other hand the loss of much time for drying, weighing, etc. The amount of phos-

phorus in the precipitate was determined colorimetrically and the zinc calculated from the amount of phosphorus found. Such an abundance of work has been done on the colorimetric determination of phosphorus that little or no difficulty is experienced by the ordinary technician in this method. Because of certain refinements and simplifications in technique the Fiske and Subbarow (16) method was used throughout this work, with only the slight alterations necessary to adapt it to the precipitate with which we were dealing.

#### *Reagents*

1. *CuSO<sub>4</sub> Solution*—1 cc. = 1.0 mg. of Cu.
2. *Sodium Citrate Solution*—100 gm. of the purified salt to 300 cc. of water.
3. *Concentrated Hydrochloric Acid*—Mallinckrodt, reagent quality.
4. *Concentrated Potassium Hydroxide*—Saturated solution.
5. *10 Per Cent Diammonium Hydrogen Phosphate*—10 gm. of the purified salt per 100 cc. of solution, kept at pH of 8 to 9 by addition of ammonia.
6. *1 Per Cent Diammonium Hydrogen Phosphate*—10 cc. of the above solution per 100 cc. made faintly pink to phenolphthalein with ammonia.
7. *Standard Phosphorus Solution*—1 cc. = 0.1 mg. of P. A solution of pure monopotassium phosphate (0.439 gm. per liter) made up in 0.1 N H<sub>2</sub>SO<sub>4</sub>.
8. *Aminonaphtholsulfonic Acid*—Dissolve 0.5 gm. of purified 1, 2, 4-aminonaphtholsulfonic acid in 500 cc. of 5 per cent sodium bisulfite solution to which have been added 5 cc. of 20 per cent sodium sulfite solution. This solution is stable for about 3 weeks if kept in the dark.
9. *Molybdate Solution I*—A 2.5 per cent solution of ammonium molybdate in 5 N H<sub>2</sub>SO<sub>4</sub>.
10. *Molybdate Solution II*—A 2.5 per cent solution of ammonium molybdate in 3 N H<sub>2</sub>SO<sub>4</sub>.

#### *Method*

Samples containing about 0.5 mg. of zinc are thoroughly ashed in an electric furnace until all the carbon is completely destroyed. The ash is taken up in HCl (1:1), the solution is filtered, and the

filtrate made to about 30 cc. in a 100 cc. Erlenmeyer flask. 5 cc. of sodium citrate solution and 2 mg. of Cu as  $\text{CuSO}_4$  are added to the solution. The pH is adjusted to 3.5 with saturated potassium hydroxide solution, with brom-phenol blue as the indicator. When the solution has been heated to boiling,  $\text{H}_2\text{S}$  is passed in immediately and this is continued until the solution becomes cool. The contents of the flask are transferred to a 50 cc. Pyrex centrifuge tube and the flask washed once with 10 cc. of sulfureted water buffered with sodium citrate and adjusted to pH 3.5. The material is centrifuged at 1500 R.P.M. or above for 5 minutes and the supernatant liquid carefully poured off and discarded. The precipitate is washed twice with 15 cc. portions of the wash water described above. The suspension is centrifuged and the clear liquid discarded after each addition of sulfureted water.

The sulfide precipitate is broken up with a fine stream of water (about 3 cc.), 0.5 cc. of concentrated HCl is added, and the tube placed in boiling water. 0.5 cc. of 30 per cent hydrogen peroxide is now added drop by drop to oxidize the sulfide. If complete oxidation does not take place in several minutes, the tube may be heated very carefully over a flame until the solution boils and clears up. The solution is now transferred quantitatively to the original Erlenmeyer flask and evaporated over a low flame to a small volume in order to insure complete destruction of the hydrogen peroxide. The solution is diluted to 30 cc., 5 cc. of sodium citrate solution are added, the pH is adjusted to 3.5, and the reprecipitation carried out as described above. The resulting precipitate is oxidized with hydrogen peroxide in HCl solution. This solution containing only copper and zinc as chlorides is evaporated nearly to dryness to remove most of the HCl. 22 cc. of water and 2.5 cc. of HCl are added and  $\text{H}_2\text{S}$  run in for 5 minutes to precipitate all of the copper. The solution and the precipitate are transferred to a 50 cc. centrifuge tube and the flask washed twice with 5 cc. portions of the same sulfureted HCl solution as was used in the precipitation of the copper. After centrifuging for 5 minutes the supernatant liquid containing the zinc is carefully transferred by decantation to the original Erlenmeyer flask which should contain no particles of copper sulfide. The precipitate is washed with 15 cc. of the wash water used in transferring the solution from the flask to the centrifuge tube. After centrifuging for 5

minutes the clear liquid is added to the solution in the original flask.

The composite solution is evaporated almost to dryness and transferred to a 15 cc. graduated centrifuge tube with water. Care must be observed to keep the volume below 5 cc. The pH is adjusted to 6.6 (brom-thymol blue) with ammonia. The contents of the tube are brought to 90° by warming in hot water, and 0.5 cc. of 10 per cent diammonium hydrogen phosphate solution is added drop by drop. An amorphous precipitate usually appears immediately, but if the zinc is present in very small amounts some time may be required for its formation. Upon standing at 90° for 30 minutes the precipitate is transformed into a finely divided crystalline precipitate.

TABLE I  
*Recovery of Zinc*

When zinc ammonium phosphate is precipitated from pure Zn solutions			From solutions containing Cu and Zn after removal of Cu			When entire method was applied to solutions of Zn, Cu, and Fe		
Zn taken	Zn recovered		Zn taken	Zn recovered		Zn taken	Zn recovered	
mg.	mg.	per cent	mg.	mg.	per cent	mg.	mg.	per cent
1.00	0.98	98.0	1.00	0.985	98.5	1.00	0.97	97.0
0.50	0.49	98.0	0.50	0.494	98.8	0.50	0.482	96.4
0.10	0.099	99.0						

The centrifuge tube is corked and after at least 4 hours it is centrifuged for 5 minutes. The liquid is poured off carefully and the precipitate washed with 5 cc. of a 1 per cent solution of diammonium hydrogen phosphate. It is finally washed with 10 cc. of 50 per cent alcohol, centrifuged, and after the wash alcohol is discarded the tube is left in an inverted position to drain.

The precipitate is dissolved in 8 cc. of 2 N H<sub>2</sub>SO<sub>4</sub> and transferred to a 50 cc. volumetric flask. This gives the proper acidity called for by the Fiske-Subbarow method. To this are added 5 cc. of Molybdate Solution II and 2 cc. of aminonaphtholsulfonic acid. After diluting the mixture to volume, the color is compared in a colorimeter with a standard prepared similarly from 2 cc. of a standard phosphate solution with 5 cc. of Molybdate Solution I rather than Molybdate Solution II. The zinc content is calcu-

lated from the amount of phosphorus found, according to the ratio of zinc to phosphorus in  $\text{ZnNH}_4\text{PO}_4$ .

Results are presented in Table I to show the recovery of zinc from solutions containing known amounts of this element. Table

TABLE II  
*Recovery of Zinc Added to Biological Materials before Ashing*

Material	Weight of sample	Zn in sample	Recovery of added Zn	Zinc
	<i>gm.</i>	<i>mg.</i>	<i>per cent</i>	<i>mg. per kg.</i>
Milk, cow.....	200	0.539		2.69
“ “ .....	400	1.10		2.75
“ “ + 1.0 mg. Zn.....	400	2.07	97.0	
Rat liver, dried at 100°.....	3	0.232		77.2
“ “ “ “ 100° .....	5	0.381		76.2
“ “ + 0.5 mg. Zn.....	3	0.72	97.6	
Alfalfa, air-dried..	50	0.599		11.98
“ + 1.0 mg. Zn.....	10	1.057	93.8	

TABLE III  
*Zinc Content of Biological Materials*

	Weight of sample	Zn in sample	Zinc
	<i>gm.</i>	<i>mg.</i>	<i>mg. per kg.</i>
Milk, cow (limits of 7 analyses)....			2.68-2.76
Lettuce, air-dried.....	10	0.315	31.5
Soy beans, “ .....	40	1.764	44.1
Timothy hay, air-dried.....	25	0.051	2.03
Green peas, canned. Dried at 100°.....	10	0.28	28.0
Range of 3 varieties.....			28.0-40.4
Bakers' yeast, air-dried.....	5	1.48	296.0
Wheat bran, “ .....	10	0.874	87.4
Whole wheat, “ .....	20	0.442	22.1
Rolled oats, “ .....	20	1.55	77.5
Beef liver, dried at 100°.....	15	0.424	283.0

I demonstrates that zinc can be recovered quantitatively as the zinc ammonium phosphate from solutions containing small amounts of zinc alone. Values were obtained from solutions containing both zinc and copper after the copper was removed as

the sulfide. The step introduced for the removal of copper did not lower the efficiency of the zinc recovery. Results obtained when the entire procedure was applied to solutions containing zinc, copper, and iron are given also. The percentage recovery is slightly lower in this case, but exceedingly good when one considers the many steps involved in this method.

In Table II are tabulated a few of the results obtained when the method was applied to biological materials to which known amounts of zinc had been added. The zinc content of a few plant and animal products is given in Table III.

#### DISCUSSION

The values which have been presented demonstrate that consistent results are obtained when the method is applied to a variety of biological materials. However, there are a number of precautions which should be taken to insure the best results. Great care must be exercised to prevent contamination from the glassware and the reagents. Pyrex glass was used in all the determinations. Reagents of the highest purity were found to be sufficiently free from zinc in most cases. Blank determinations were made frequently to check any possible source of contamination.

The maximum temperature used for ashing should not exceed 500° and the time of ashing should be reduced to a minimum. In the event of incomplete oxidation the unoxidized portion should be returned to the furnace for further ashing. The addition of  $\text{CaCO}_3$  to materials which ash with difficulty aids greatly in decreasing the ashing time. Its use is somewhat limited due to the fact that large amounts of Ca interfere in subsequent reactions. Alfalfa, for example, which may contain as high as 3 to 4 per cent of CaO, gave considerable trouble in early attempts to analyze it for zinc. The zinc content is very low, necessitating the use of large samples and thus bringing considerable quantities of calcium into the ash extract. It was found that this amount of Ca could not be kept in solution at pH 3.5 in the volume of solution called for. Rather than lengthen the procedure by the addition of a step for the removal of Ca, the volume of the ash extract was increased to 200 cc. for the first precipitation. This was centrifuged in a 250 cc. cup and the bulk of the supernatant liquid drawn off by



suction, the remainder transferred to a 50 cc. cup, and the regular procedure followed. In the event that equipment is not available for centrifuging this amount of material the sulfides may be allowed to settle overnight and the supernatant liquid drawn off.

It is advisable to have the solution boiling at the time the hydrogen sulfide is run in as a more granular precipitate is thus produced. After oxidation of the sulfides in the centrifuge tube the solution is returned to the original Erlenmeyer flask. This contains small amounts of hydrogen sulfide which must be expelled before the solution is returned to avoid precipitation under unfavorable conditions.

Zinc chloride is volatile at relatively low temperatures, and for this reason great care must be exercised in the various evaporations. Complete evaporations are in no case necessary; therefore, the evaporation should always be stopped while a small amount of solution remains. The 10 per cent ammonium acid phosphate solution is kept at pH 8 to 9 with ammonia to insure the presence of the diammonium salt. Upon addition of this to the zinc solution, which has a pH of 6.6, the acidity is lowered to the desired pH of 7, and at the same time the phosphate functions as a buffer to hold this acidity which is quite necessary as the heating for 30 minutes expels ammonia. In the event that the pH of the resulting solution is not 7, it is so adjusted with dilute ammonia or HCl. The 1 per cent ammonium hydrogen phosphate solution used for washing the zinc precipitate is kept just pink to phenolphthalein, thus allowing a pH close to neutral.

In the phosphorus determination any volume may be used in order to obtain the desired intensity of color for comparison with the standard. Care should be taken, however, to maintain the acid concentration close to 0.6 N  $\text{H}_2\text{SO}_4$ . With a little experience the most desirable volume for the dilution of the solution of the zinc ammonium phosphate precipitate can be chosen and the correct amount of 2 N  $\text{H}_2\text{SO}_4$  easily calculated.

#### SUMMARY

1. A method has been outlined for the determination of small amounts of zinc in biological materials.

2. The method is adaptable to amounts of zinc varying from 0.1 to 2.0 mg.

3. A table (Table III) has been included to show the results of determinations on several biological products.

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## COMPARATIVE STUDIES OF THE METABOLISM OF THE AMINO ACIDS

### V. THE OXIDATION OF PHENYLALANINE AND PHENYLPYRUVIC ACID IN THE ORGANISM OF THE RABBIT\*

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The problems of the intermediary metabolism of the aromatic amino acids of the protein molecule, tyrosine, and phenylalanine, have been investigated by a number of experimental methods, of which perfusion of the acids and their possible intermediary catabolites through surviving organs and studies of their fate in the human organism in alkaptonuria or in the organisms of normal laboratory animals have been most productive. Excellent critical evaluation of this work is available in the summaries of Dakin (1), in 1922, and of Mitchell and Hamilton (2), in 1929. The data obtained have seldom been of a quantitative nature since methods for the exact determination of the various substances, whose existence as intermediary catabolites has been proved, have not been available. The results have had a qualitative significance, therefore, rather than a quantitative one, since the methods for the most part have involved isolation, a procedure inadequate from the quantitative view-point. In the present investigation an attempt has been made to study the excretion of some of the intermediary metabolites quantitatively and to determine the extent to which those reactions, which result in the formation of phenyl derivatives not readily oxidized further, occur after the feeding or injection of phenylethylamine, phenylalanine, and phenylpyruvic acid to rabbits.

\* A preliminary report of part of this work was presented before the Twenty-fourth annual meeting of the American Society of Biological Chemists at Chicago (*J. Biol. Chem.*, **87**, p. lvi (1930)).

## EXPERIMENTAL

Rabbits were given phenylalanine and related compounds orally or subcutaneously and the urine excreted during the period immediately following was examined for possible intermediate products, chiefly phenylpyruvic, phenylacetic, and benzoic acids, and the conjugation products of the last two with glycine.

Male rabbits were maintained on constant weighed diets of cabbage and oats over the period of study which in some cases exceeded 3 months in duration. Since the volume of urine excreted by the rabbit is so small that it is difficult to make a series of analyses which require considerable amounts of urine on 24 hour specimens, 48 hour samples were obtained and analyzed. Hydrolysis of hippuric or phenaceturic acid prior to the analysis was prevented by the addition of dilute nitric acid. Determinations of creatinine were made in order to secure a check on the completeness of the collection of the 48 hour samples.

Equal amounts of the substances under investigation were administered on 2 consecutive days, usually through a stomach tube. Two 2 gm. portions of phenylalanine or equivalent amounts of sodium phenylpyruvate were given during the experimental periods. Partial neutralization of the amino acid was necessary in order to dissolve it in a volume convenient for administration (usually 40 cc.). In a few experiments subcutaneous injection of the compound to be studied was employed in order to exclude possible chemical changes due to the activities of the intestinal microorganisms.

The phenylpyruvic acid, synthesized in this laboratory by the method of Hemmerle (3), because of the instability of the free acid was purified as the sodium salt and fed in this form. Since the sodium salt of the acid contains 1 molecule of water of crystallization, 1.25 gm. of the salt are equivalent to 1 gm. of the free acid and also to 1 gm. of phenylalanine. Very dilute solutions of the acid or salt gave the characteristic green color with ferric chloride. All of the preparations were examined for the presence of phenylacetic acid with negative results.

*Methods of Analysis*

The Wayne modification (4) of the Steenbock sublimation method for benzoic and phenylacetic acids was used in all the

quantitative experiments. A ground glass joint between the U-tube and the sublimation tube was found to be an improvement over the rubber stopper connection used by Wayne, since removal of the sublimate from the horizontal part of the U-tube by heating is difficult without burning the rubber stopper of the Wayne apparatus. Difficulty was experienced in applying this method to the determination of small quantities of benzoic acid and phenylacetic acid in mixtures. In experiments with known amounts of pure substances, the total weight of sublimate checked fairly well with the total amounts of acids taken, but the calculated proportions of benzoic and phenylacetic acids were frequently inconsistent. Although the method has been useful as indicating the approximate values, we have found it desirable to supplement the Wayne method with other determinations.

Phenylpyruvic acid is converted to phenylacetic acid under the conditions of the Wayne method and the phenylacetic acid derived from it appears in the sublimate. In experiments with different preparations of sodium phenylpyruvate, about 75 per cent of the theoretical amount of phenylacetic acid was obtained in the sublimate. It was at first believed that the observed formation of phenylacetic acid was due to the presence of an oxidizing agent (hydrogen peroxide) during the hydrolysis. Experiments in which the hydrogen peroxide was omitted, however, demonstrated that this was not the case. In one experiment, for example, 0.250 gm. samples of sodium phenylpyruvate (equivalent to 0.165 gm. of phenylacetic acid) were subjected to the procedure of the Wayne method both with and without the presence of hydrogen peroxide during hydrolysis. By the original method, 0.117 and 0.119 gm. of sublimate were obtained in duplicate determinations, while without the presence of the peroxide, the weights were 0.052 and 0.050 gm. The sublimed material in these experiments was identified as phenylacetic acid by recrystallization and determination of the melting point. From these and other similar experiments, we may conclude that the conversion of phenylpyruvic acid to phenylacetic is not complete by this method even when the peroxide is present and that some formation of phenylacetic acid occurs even in the absence of an oxidizing agent. Phenylalanine gave no sublimate in the Wayne method.

The behavior of phenyllactic acid<sup>1</sup> was also studied. Samples of 0.3 gm. were subjected to the procedure of the Wayne method both with and without the addition of hydrogen peroxide. In the presence of the oxidizing agent, the sublimed acid obtained amounted to 33.7 and 26.4 per cent of the amount of phenylacetic acid which could be derived by oxidation, while in the absence of peroxide, the amount of sublimate was almost negligible, 3.4 and 3.1 per cent of the value expected if complete conversion to the acetic acid derivative had occurred. It is evident that the amount of sublimable material obtained in the Wayne procedure from phenyllactic acid is much less than that obtained from phenylpyruvic acid especially in the absence of peroxide.

The method described by Griffith (5) for the determination of hippuric acid was also employed in several series of experiments. This method, which was originally developed for hippuric acid, also determines any other ether-soluble nitrogenous compounds (*e.g.* phenaceturic acid) not decomposed by alkaline hypobromite. Both phenylacetic and benzoic acids are determined if conjugated with glycine while neither free benzoic, phenylacetic acid, nor the products of their conjugation with non-nitrogenous substances (*e.g.* glycuronic acid) are determined by this procedure. Since we were concerned not only with hippuric acid but also with phenaceturic acid, which is less readily soluble in ether than hippuric acid, the ether extraction was carried out for 2 hours, in order to be certain that all of the phenaceturic acid was extracted.

In the last series of experiments, the Kingsbury-Swanson modification (6) of the Folin-Flanders method for total benzoic acid (free and combined) was used in addition to the other methods. *Total phenylacetic acid* is obviously included in this determination since it and its conjugated derivatives react similarly to benzoic acid. The oxidizing agents employed will also convert some phenylpyruvic acid if present to phenylacetic acid which will be extracted and titrated. Any other chloroform-soluble acids present in the hydrolysate will be determined also.

Because of the failure of the Wayne sublimation method to

<sup>1</sup> The phenyllactic acid used in these control experiments was prepared by Dr. Julius White. A grant from the Faculty Research Fund made possible the preparation of this substance.

estimate with sufficient accuracy small amounts of benzoic and phenylacetic acids, a modification of the method of Virtanen and Pulkki (7) for the estimation of volatile acids was developed. This method depends upon the principle utilized in the well known Duclaux method for the determination of the volatile fatty acids, that a *definite fraction* of a volatile substance is distilled from an aqueous solution in a *given time* under *specified conditions*. Variations in concentration within wide limits and the presence of other volatile substances do not affect this constant rate of distillation. In this method 200 cc. of the solution to be analyzed are placed in a 300 cc. Erlenmeyer flask fitted to a condenser and the rate of boiling so adjusted that 100 cc. of distillate are collected in 1 hour. The distillate and original solution are titrated with standard alkali. Under these conditions according to Virtanen and Pulkki, 4.8 per cent of the total phenylacetic acid and 17.2 per cent of the total benzoic acid present are found in the distillate. In our experiments in which the amounts of phenylacetic acid ranged from 60 to 100 mg., we obtained values ranging from 4.7 to 5.0 per cent, and with 40 to 125 mg. of benzoic acid, values from 16.9 and 17.3 per cent. With mixtures of these two acids, the amount of each may be calculated from a formula involving the titration values of the distillate and the original solution. In Table I are presented some representative results of analyses of known mixtures of the pure acids, which show that the method can be used with a fair degree of accuracy, even when small amounts of the acids only are present.

In order to apply this method to the analysis of urine, the benzoic and phenylacetic acids must first be separated from the other acids present. This is done quite efficiently by the sublimation method of Wayne. The alcoholic solution of the sublimate may then be titrated for the Wayne determination and the acids then extracted with ether from the acidified titration mixture. The ether extract is evaporated, the residue is dissolved in water, and the distillation is carried out as above described. It is possible in this way to determine the phenylacetic and benzoic acids by two methods with the use of the same sample of urine. A mixture of known amounts of benzoic and phenylacetic acids in solution was titrated and the mixture of the free acids recovered from this solution of their salts by ether extraction of the acidified

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solution was distilled according to the Virtanen-Pulkki procedure. Typical results are presented in Table II. In both Tables I and II, there is observed a tendency for the phenylacetic acid values to be slightly high and for those for benzoic acid to be slightly low.

TABLE I

*Estimation of Benzoic and Phenylacetic Acids in Mixtures, by the Method of Virtanen and Pulkki*

Mixture used		Titration of distillate	Titration of original solu- tion	Benzoic acid calculated	Phenylacetic acid calculated
Benzoic acid	Phenylacetic acid				
mg.	mg.	cc. 0.06 N alkali	cc. 0.06 N alkali	mg.	mg.
16	16	0.53	4.97	14.8	17.3
16	80	0.96	14.35	13.3	82.8
40	80	1.68	18.20	39.9	79.4
40	80	1.63	18.25	37.0	83.1
50	50	1.79	15.32	51.7	46.6
60	60	2.05	18.45	57.2	61.8
80	40	2.47	18.92	77.0	43.0
80	16	2.30	15.30	77.5	17.8
100	50	3.21	23.50	102.8	45.6
12	20	0.45	4.92	10.6	21.7

TABLE II

*Recovery of Benzoic and Phenylacetic Acids from Solutions of Their Salts by the Modified Virtanen-Pulkki Procedure*

Present		Recovered	
Benzoic acid	Phenylacetic acid	Benzoic acid	Phenylacetic acid
mg.	mg.	mg.	mg.
20	20	14.0	21.0
15	25	12.0	22.0
20	100	16.5	97.8
100	20	89.5	23.0

These errors, however, are not sufficiently great to affect the value of data obtained by this method in animal experiments, especially if the values are considered in relation to the values obtained by the other methods discussed.

For the identification of phenylpyruvic acid in the experimental



urines, the semicarbazone derivative was prepared. The urines, acidified and shaken with Lloyd's reagent to remove as much pigment as possible, were saturated with solid ammonium sulfate and extracted with ether until the ether extracts no longer gave a green color with dilute ferric chloride solution. The acid was then removed from the ether extracts by shaking with a small volume of dilute alkali. In this way it was possible to concentrate the phenylpyruvic acid from a considerable volume of urine. On acidification of the alkaline solution and addition of semicarbazide hydrochloride, the semicarbazone was obtained.

#### DISCUSSION

Six rabbits were used in this series in which seven experiments with *l*-phenylalanine,<sup>2</sup> eight with *dl*-phenylalanine, three with sodium phenylpyruvate, and one with phenylethylamine were carried out. In addition, as controls of the experimental procedure, benzoic acid was administered in two experiments and phenylacetic acid in three. Results typical of those obtained in all the experiments are presented in Tables III and IV. The figures presented for the normal control periods represent the averages of a large number of 48 hour periods since each experimental period was preceded and followed by at least one, usually two, control periods. Thus the control figures for Rabbits 3 (Table III) and 4 (Table IV) are averages of the values obtained in eighteen and twenty-one periods respectively.

Increases in the weight of the sublimate of the Wayne method were obtained after the oral or subcutaneous injection of *dl*-phenylalanine (Table III). The ingestion of *l*-phenylalanine, on the other hand, resulted in slight increases only in the weight of the sublimed acids. In three experiments with Rabbit 2 (only one of which is recorded in Table III) in which the natural optical isomer of phenylalanine was fed, the change in the weight of the sublimate was too small to be of significance. With

<sup>2</sup> The *l*-phenylalanine was presented to us by Dr. Seiichi Izume of the Central Laboratory of Dairen, Manchuria, to whom we wish to express our appreciation. This product, obtained from wheat gluten as a by-product in the preparation of the condiment, ajinomoto, contained a small amount (about 2 per cent) of tyrosine as determined colorimetrically by the modified Millon reaction of Folin and Ciocalteu.

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other animals (e.g., Rabbit 3 (Table III)), the increase after *l*-phenylalanine was more marked. By far the greatest increases in the amount of sublimate were noted after oral administration of the sodium salt of phenylpyruvic acid. Increases in phenac-

TABLE III  
*Influence of Phenylalanine and Phenylpyruvic Acid on Excretion of Phenylacetic Acid*

All results are calculated as the amounts of phenylacetic acid excreted in 48 hour periods. Unless otherwise indicated, the acids were administered orally.

Substance administered	Griffith method	Wayne method		
		Hydrogen peroxide*	Weight of sublimate	Titration of sublimate
	gm.		gm.	gm.
Rabbit 2, weight 3.2 kilos				
Average control.....	0.161	+	0.151	0.183
		—	0.170	0.165
<i>l</i> -Phenylalanine, 4.0 gm.....	0.149	+	0.170	0.163
		—	0.129	0.140
<i>dl</i> -Phenylalanine, 4.0 gm.....	0.347	+	0.566	0.625
		—	0.296	0.318
Rabbit 3, weight 3.7 kilos				
Average control.....	0.178	+	0.159	0.167
		—	0.147	0.158
<i>l</i> -Phenylalanine, 4.0 gm.....	0.219	+	0.311	0.318
		—	0.288	0.293
<i>dl</i> -Phenylalanine, 4.0 gm.....	0.261	+	0.638	0.635
		—	0.298	0.280
“            4.0 “ subcutaneously.	0.246	+	0.548	0.588
		—	0.337	0.321
Sodium phenylpyruvate, 5.0 gm.....	0.677	+	1.003	0.972
		—	0.867	0.805

\* A plus sign indicates that hydrogen peroxide was used in the Wayne method and a negative sign that the peroxide was omitted.

eturic (or hippuric) acid excretion as measured by the Griffith method were usually obtained in the same periods in which the weight of sublimed acids showed increase. Calculations by the Wayne method indicated that these increases in sublimate were

due to the presence of phenylacetic acid, but further and more satisfactory evidence of this is afforded by the data obtained by the

TABLE IV

*Excretion of Phenylacetic and Benzoic Acids after Oral Administration of Benzoic and Phenylacetic Acids and of Phenylalanine and Phenylpyruvic Acid*

Rabbit 4, weight 3.2 kilos. All results are expressed in terms of gm. of phenylacetic acid excreted in 48 hour periods unless otherwise indicated.

Substance fed	Griffith method	Kingsbury-Swanson method	Wayne method. Sublimate			
			Hydrogen peroxide*	Titration of sublimate	Virtanen distillation of acids in sublimate	
					Phenylacetic acid	Benzoic acid
	gm.	gm.		gm.	gm.	gm.
Average control period.....	0.191	0.242	+	0.181	0.068	0.058
			-	0.165	0.068	0.057
Benzoic acid, 0.400 gm.....	0.527†	0.601†	+	0.458†	0.086	0.311
			-	0.400	0.082	0.301
Phenylacetic acid, 0.440 gm.....	0.523	0.562	+	0.531	0.352	0.100
			-	0.493	0.335	0.089
“ “ 0.440 “ .....	0.542	0.605	+	0.557	0.400	0.071
			-	0.567	0.416	0.076
<i>l</i> -Phenylalanine, 4.0 gm.....	0.352	0.444	+	0.392	0.211	0.059
			-	0.366	0.219	0.056
“ 4.0 “ .....	0.266	0.395	+	0.348		
			-	0.265	0.137	0.089
<i>dl</i> -Phenylalanine, 4.0 gm.....	0.224	0.771	+	0.669	0.435	0.089
			-	0.372	0.242	0.063
“ 4.0 “ .....	0.234	0.711	+	0.551	0.405	0.097
			-	0.296	0.162	0.082
Sodium phenylpyruvate, 5.0 gm.....	1.015	1.364	+	1.370	1.205	0.051
			-	1.232	0.977	0.039

\* A plus sign indicates that hydrogen peroxide was used in the Wayne procedure; a negative sign, that hydrogen peroxide was omitted.

† Calculated as benzoic acid.

of the modification of the Virtanen and Pulkki method previously described (Table IV).

In order to assure ourselves further as to the reliability of the results obtained by the Virtanen distillation procedure, control experiments in which benzoic and phenylacetic acids were fed were carried out (Table IV). In each case, the results were those anticipated; *i.e.*, a marked increase in amount of the benzoic acid content of the sublimate when benzoic acid was ingested, and a similar increase in phenylacetic acid after the administration of phenylacetic acid. We believe that the results of these animal experiments together with the control experiments on the accuracy of the method as applied to known mixtures of these acids already described indicate that the method may be depended upon to give significant results.

In the experimental periods in which phenylpyruvic acid was fed, the results obtained with the Virtanen procedure showed that the increases in the amount of the sublimate were almost entirely accounted for by the increase in the phenylacetic acid fraction. Since by this method, no significant increases in the benzoic acid of the sublimate could be demonstrated, the increased values in the Griffith method would appear to have been due to the excretion of phenaceturic acid rather than hippuric. The increase following the ingestion of phenylpyruvic acid was especially marked, the phenaceturic acid (calculated as phenylacetic acid) excretion being 1.015 gm. in a 48 hour period (Table IV, calculated from determinations by the method of Griffith). Confirmation of these results was obtained by the isolation of phenaceturic acid from the urine of some experimental periods. Thus in one experiment, after 2.5 gm. of sodium phenylpyruvate had been fed for 2 successive days, it was possible to isolate from the urine 0.4 gm. of phenaceturic acid which melted at 142°. From the isolated phenaceturic acid, phenylacetic acid with a melting point of 75.5° was obtained after hydrolysis. The evidence indicates that under our experimental conditions phenylpyruvic acid is converted in part to phenylacetic acid which is conjugated with glycine and excreted as phenaceturic acid in the urine. The evidence of the formation of phenaceturic acid after the administration of phenylalanine is not so convincing. In five experiments in which *dl*-phenylalanine was administered and the excretion followed by the Griffith method, an increase in phenaceturic acid by this method was observed in three instances (Table III) while

in two experiments (Table IV), the values were only slightly higher than those of the control periods. With *l*-phenylalanine the results in one experiment (Table IV) clearly indicated an excretion of phenaceturic acid, with slightly increased excretion in two others, and no evidence of any change in a fourth (Table III). It should be noted that one of these positive results was observed after subcutaneous injection.

After the ingestion of phenylalanine or phenylpyruvic acid, increases in the values obtained by the Wayne method (either in the weight of the sublimate or in the amounts calculated from the titration of the acidity of the sublimate) were always greater than the increases of phenylacetic as phenaceturic acid (Griffith method) for the same experimental period. It was apparent that some other substance also gave rise to phenylacetic acid in the Wayne procedure. An excretion of phenylacetic acid either free or in conjugation with a non-nitrogenous substance (*e.g.* glycuronic acid) would explain this difference. However, the conjugation of phenylacetic acid with glycine would seem to be nearly quantitative in our experimental animals since in neither of the two experiments reported in which phenylacetic acid was fed (Table IV) was there observed a greater increase in the amount of total sublimable organic acid (Wayne method) than in the phenylacetic acid as phenaceturic acid (Griffith method).

It seems probable, therefore, that the presence in the urine of phenylpyruvic acid, which had previously been shown to be converted in large part to phenylacetic acid in the oxidation incidental to the Wayne method, was responsible for the different values obtained by the Wayne and Griffith methods. One of us (8) has presented evidence obtained by colorimetric methods of the excretion of significant amounts of this substance after the administration of phenylalanine. In the present series of experiments, we have been able to prepare the semicarbazone and hydrazone of phenylpyruvic acid from a number of urines secreted during experimental periods in which *dl*-phenylalanine, *l*-phenylalanine, and sodium phenylpyruvate were fed. Less direct evidence was depended upon to prove the presence of the same keto acid in other experimental urines. This evidence was supplied partly by the ferric chloride test which was positive for the urines of the experiments in which the keto acid was isolated as well as for the

urines of all other experiments in which phenylalanine or phenylpyruvic acid was fed or injected. In many experiments in which a strongly positive ferric chloride test was given by the urine, an increased value in the creatinine determination was also observed. The apparent increase of this urinary constituent was due, no doubt, to the presence of phenylpyruvic acid, which previous workers in this laboratory (8) found to be a source of color production in this colorimetric determination. There is little doubt then, that phenylpyruvic acid, present in the urines of experimental periods showing increased sublimates, was the chief source of the phenylacetic acid determined in the Wayne method and not arising from phenaceturic acid.

Approximate estimates of the amounts of the keto acid excreted may be made from the data. Possible sources of the increase of phenylacetic acid in the sublimate of an experimental period were phenylpyruvic acid, phenyllactic acid, and phenaceturic acid. The increase of phenylacetic acid derived from phenaceturic acid (Griffith method) may be subtracted from the total increase of phenylacetic acid, to give as the difference the extra phenylacetic acid derived from phenylpyruvic acids. Estimations by this method show that the largest amounts of the keto acid were excreted after the ingestion of the acid itself, the next largest after *dl*-phenylalanine, and the least after *l*-phenylalanine. The intensities of the green color in the ferric chloride test were in agreement with those estimations. Attempts to eliminate the influence of the keto acid on the sublimate were made by omitting the use of the oxidizing reagent in the procedure. The results of these experiments were of little value from the quantitative standpoint since appreciable amounts of phenylacetic acid were derived from phenylpyruvic acid even under these conditions. However, the difference in the results obtained with and without the use of the oxidizing agent (Tables III and IV) demonstrates that a substance yielding phenylacetic acid on oxidation was present in considerable amounts and thus offers indirect evidence of the presence of phenylpyruvic acid<sup>3</sup> confirmatory of the other evidence of the presence of this catabolite.

<sup>3</sup> It is of course possible that some of this phenylacetic acid was derived not from phenylpyruvic acid, but from phenyllactic acid formed in intermediary metabolism. In view of the relatively smaller amounts of sub-

In one experiment (Table III) *dl*-phenylalanine was injected subcutaneously. The result obtained was in all respects very similar to those obtained in the corresponding feeding experiments. This result shows that the changes observed in the feeding experiments were not due to microorganisms of the intestine. In one experiment not shown in the tables (*cf.* also Table V), sodium phenylpyruvate was injected subcutaneously and the urine analy-

TABLE V

*Effect of Oral Administration of Phenylethylamine Hydrochloride on Excretion of Phenylacetic and Benzoic Acids*

Rabbit 6, weight 3.0 kilos. Data are expressed as gm. of phenylacetic acid excreted in 48 hour periods, except where otherwise indicated.

Period	Griffith method	Wayne method. Titration of sublimate	Virtanen method	
			Phenylacetic acid	Benzoic acid
	gm.	gm.	gm.	gm.
Control.....	0.234	0.162	0.072	0.050
" .....	0.213	0.147	0.077	0.047
Experimental*.....	1.166	1.430	1.370	0.022
Control.....	0.904	1.147	1.083	0.020
" .....	0.136	0.147	0.117	0.021
" .....	0.168	0.149	0.082	0.046
Experimental†.....	0.243	0.601	0.509	0.051
Control.....	0.123	0.111	0.060	0.028
" .....	0.135	0.149	0.090	0.033

\* 1.9 gm. of phenylethylamine hydrochloride were fed daily on 2 successive days.

† 2.5 gm. of sodium phenylpyruvate were injected subcutaneously on 2 successive days.

zed by the Griffith method. The phenaceturic acid excreted in a preceding control period was 0.301 gm., while after the injection it was 0.488 gm. This increase is much less than those which

limate obtained from phenyllactic acid in check experiments with the Wayne method as already described *particularly in the absence of peroxide*, we believe that little of the phenylacetic acid was derived from this source. Further evidence in support of this belief that phenylpyruvic acid is the more important intermediary catabolite is the parallelism between the intensity of the ferric chloride reaction and the amount of sublimate. Phenyllactic acid gives only a faint yellow-green color with ferric chloride.

occurred in the two experiments in which the same amount of phenylpyruvic acid was given orally, and suggests a formation of phenylacetic acid in the gastrointestinal canal from phenylpyruvic acid in the latter experiments. Some formation of phenaceturic acid appears to have occurred after injection of the keto acid, however. More complete data from other injection experiments will be necessary to permit an explanation of this difference.

In view of the possibility of decarboxylation, suggested by Thomas (9) as a possible intermediary step in amino acid catabolism, by which phenylethylamine would be derived from the phenylalanine, a study of the behavior of this amine in metabolism was made. Guggenheim and Löffler (10) have demonstrated the oxidation of this substance to phenylacetic acid in the body but no quantitative study appears to have been reported. Phenylethylamine hydrochloride was fed to a rabbit and analyses similar to those already discussed were carried out. The results (Table V) showed a marked increase in the excretion of phenylacetic acid by all the analytical methods used. This increased excretion as measured by the Wayne sublimation and Virtanen distillation methods was approximately the same, 2.28 and 2.30 gm. respectively. This corresponds to a recovery of approximately 78 per cent of the phenylacetic acid which could be derived from the oxidation of the amine to phenylacetic acid. By far the greatest part of the "extra" phenylacetic acid was eliminated as an ether-soluble nitrogenous compound (*i.e.*, phenaceturic acid) as shown by the analyses in which the Griffith method was used. 1.76 gm. of "extra" phenylacetic acid as determined by this method were excreted corresponding to about 60 per cent of the amount of phenylacetic acid which could be derived by oxidation of the amine fed. That the increased excretion as shown by the Virtanen method was due to the presence of phenylacetic was proved by the isolation of 0.4 gm. of phenylacetic acid with a melting point of 76° from a part of the solution distilled. No unchanged phenylethylamine could be detected in the urines. When solutions of the amine were subjected to the procedures of the Wayne and Griffith methods, no appreciable amounts of the substances determined by these methods were formed. In view of these results, phenylethylamine would appear to be readily converted to phenylacetic acid or its derivatives in the animal body.



Therefore, if, as suggested by Thomas (9), phenylalanine undergoes decarboxylation prior to deamination, complete oxidation of the amino acid would not occur.

One result of these experiments has been the demonstration of the conversion of phenylalanine to phenylpyruvic acid and the excretion of the latter in the urine, a result in agreement with previous data obtained by colorimetric methods in this laboratory (8) and with the work of Kotake and his coworkers (11). The most reasonable explanation of this failure of complete oxidation of the benzene ring is that, as a result of the deamination of phenylalanine, the keto acid is formed so rapidly that the disruption of the benzene nucleus cannot be effected and the intermediate product is excreted. A criticism may be raised in both series of experiments that the amounts of amino acids ingested were beyond the amounts which would be present in the organism under normal conditions of metabolism. Such a criticism may be warranted as far as concerns the experiments of the Japanese investigators, in which 9 gm. of phenylalanine were administered daily to rabbits of about 2.5 kilos of body weight and the administrations were continued for several successive days in some experiments. In our own experiments, however, considerably less than 1 gm. of phenylalanine per kilo of body weight was fed for 2 successive days only. In a further attempt to answer this possible criticism, a series of experiments was undertaken to determine the minimal amount of phenylalanine, which when fed to a rabbit of about 3 kilos of body weight would result in the excretion of phenylpyruvic acid in such amounts that it could be detected by the ferric chloride color reaction. A strongly positive test was obtained from urine collected 1 hour after the administration of 0.150 gm. of *dl*-phenylalanine, but no test could be observed after administration of smaller amounts of the amino acid. The ingestion of much larger amounts of *l*-phenylalanine was required in order to obtain a positive ferric chloride test in the urine. In similar experiments, phenylpyruvic acid could be readily detected colorimetrically in the urine 1 hour after feeding 0.160 gm. of phenylpyruvic acid as the sodium salt.

In our experiments much of the  $\alpha$ -keto derivative formed from phenylalanine was excreted without further oxidation, but a small portion appears to have been converted to phenylacetic acid and excreted as phenaceturic acid. Although the formation of phenyl-

acetic acid in intermediary metabolism does not appear to have been demonstrated previously for phenylalanine, similar reactions which involved amino acids foreign to the body have been reported (12, 13). It is possible that the presence of amounts of phenylalanine, greater than those which might be expected to be formed in the course of the normal metabolism, may have resulted in a series of intermediary reactions, distinct from the normal, and that in our experiments, we were concerned essentially with an amino acid foreign to the organism. The detection of increased amounts of the acetic acid derivative after the administration of small amounts of phenylalanine would aid materially in determining whether phenylacetic acid may originate from phenylalanine in normal metabolism, but unfortunately, adequate methods for the determination of small amounts of phenylacetic acid or its conjugated derivatives are not available. It may be pointed out, however, that phenaceturic acid has been isolated as a constituent of normal urine of many species (14, 15). Our analyses of the sublimable organic acids of the control periods by the modified Virtanen procedure (Table IV) have indicated that these organic acids may consist of phenylacetic and benzoic acids in nearly equal amounts. Since the solubilities of hippuric and phenaceturic acids in organic solvents are very similar, it seems probable that of the material in the normal urine determined and calculated as hippuric acid, a considerable part may be phenaceturic acid.

Finally it should be pointed out that these experiments offer no evidence, positive or negative, concerning the formation of phenyllactic acid as an intermediary catabolite in the metabolism of phenylalanine. Certain observers (16) have isolated phenyllactic acid in the urine after the administration of phenylpyruvic acid and have thus demonstrated a reversal of the normal oxidative process. Our results have shown that in rabbits phenylpyruvic acid may be excreted in significant amounts after the administration of both phenylalanine and phenylpyruvic acid and that under the conditions of our experiments some of this phenylpyruvic acid may be oxidized to phenylacetic acid. Our experiments emphasize anew that if ready and complete oxidation of the benzene ring in phenylalanine is to be effected, the initial disruption of the ring must occur before the side chain is oxidized.

## SUMMARY

1. A study has been made of the application of the method of Virtanen and Pulkki (7) to the quantitative determination of phenylacetic and benzoic acids or their conjugated derivatives in the urine in connection with the intermediary metabolism of phenylalanine.

2. On a diet of cabbage and oats, rabbits excreted approximately equal amounts of phenaceturic and hippuric acids as determined by the method of Virtanen and Pulkki.

3. Evidence of the presence of considerable amounts of phenylpyruvic acid in the urine of rabbits after oral administration or subcutaneous injection of 2.0 gm. of phenylalanine or an equivalent amount of phenylpyruvic acid was obtained. More of this keto acid was excreted after the administration of *dl*-phenylalanine than after the administration of *l*-phenylalanine.

4. A slight increase in the excretion of phenaceturic acid was observed in some experiments after oral administration or subcutaneous injection of 2.0 gm. of phenylalanine and a greater increase after the administration of an equivalent amount of phenylpyruvic acid. Under the same conditions no significant increase of hippuric acid excretion was observed.

5. These results would indicate that in the case of phenylalanine,  $\alpha$  oxidation may follow oxidative deamination to give products which are not oxidized further in the organism. It is usually assumed that following oxidative deamination of phenylalanine, opening of the benzene ring occurs before further chemical changes in the side chain. This is apparently not the only path of catabolism open. The results of the present study indicate that the benzene ring of phenylalanine is oxidized less readily than has usually been assumed.

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## THE DETERMINATION OF CHOLIC ACID IN BILE\*

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In attempting to apply the method described by Gregory and Pascoe (1929) to the determination of bile acids in bile, considerable difficulty was experienced because of the weakness of the blue color developed by cholic acid. Later it was found that because of a misprint in the original paper only one-fifth of the required amount of cholic acid was being used. Before this was known several attempts had been made to increase the color by means of higher concentrations of  $\text{H}_2\text{SO}_4$  and furfural. The result was a deeper color and a marked rise in absorption of the monochromatic red light with which the colorimetric comparisons were made. The favorable effect of the increased concentration of the reagents led to a detailed investigation of principal factors concerned with the development of the color, and when these were adjusted so as to yield maximal values, the sensitivity of the method was more than doubled, while the best results by the new technique are obtained with one-fourth of the amount of bile acid required for the old procedure. The improved sensitivity is particularly advantageous in the analysis of blood and of pathological human bile where low concentrations of cholic acid are frequently encountered. An additional advantage is the reduction in the time of heating from 30 minutes to 8 minutes. Further evidence of the specific nature of the reaction between cholic acid and the furfural- $\text{H}_2\text{SO}_4$  reagent is presented, including a comparison of

\* This paper is taken from the thesis of Mr. Reinhold, to be presented in partial fulfillment of the requirements for the degree of Doctor of Philosophy to the Faculty of the Graduate School of the University of Pennsylvania.

desoxycholic acid with cholic acid. The effect of conjugation on the color formed by cholic acid has also been investigated. The results of these studies have been incorporated into the procedure described in the following section.

### *Method*

The preliminary treatment of the bile depends on the nature and amount of protein present. Riegel, Johnston, and Ravdin (1932) observed that the small amount of protein in dog bile did not interfere with their modified application of the Gregory and Pascoe procedure, and we have found this to be true also of the technique described in this paper. However, human gallbladder bile contains significant amounts of protein that form an interfering color and cause high results. If this protein is present in human hepatic bile, the amount is too small to have a recognizable effect, and as in dog bile the removal of protein is not essential.

*Procedure for Dog Bile or Human Hepatic Bile*—Gallbladder bile from the dog is diluted 250 to 500 times with distilled water; hepatic bile 50 to 100 times, or less if the rate of flow is rapid.

1 cc. of bile is diluted as described, and 1 cc. of the diluted bile is measured into a test-tube (18 mm. diameter). To this are added 1 cc. of freshly diluted 0.9 per cent (by volume) aqueous furfural and 6 cc. of 16 N  $\text{H}_2\text{SO}_4$ . The contents of the tube are mixed thoroughly by tapping or by stirring. The resulting solution is 12 N  $\text{H}_2\text{SO}_4$  containing 1.3 mg. of furfural per cc. The tubes are then stoppered loosely with rubber stoppers, and are heated in a water bath at 70° for 8 minutes. For the best results, the temperature should not vary more than 1–2°. Cooling is accomplished by placing the tubes in tap water for 2 minutes.

1 cc. of a standard solution of sodium cholate in water, containing 0.215 mg. (0.5 mm solution), is treated like the unknown. Colorimetric comparison is made in monochromatic light of about 6000 to 6900 Å. with the unknown set at 15.0 mm.

*Procedure for Human Gallbladder Bile*—1 cc. of freshly collected bile is added slowly to about 10 cc. of 95 per cent alcohol in a test-tube. After mixing, the contents of the tube are warmed to the boiling point of the alcohol in a water bath. The precipitate of protein is removed preferably by centrifugation since filtration causes a small loss, and the alcohol is decanted into a 25 cc. volumet-

ric flask. The precipitate is extracted with 10 cc. of alcohol by heating, centrifuging, and decanting as before. Usually the precipitate still contains small quantities of cholic acid, and for careful work a second extraction with 5 cc. of alcohol is needed. The volume is then made to 25 cc. with alcohol. Specimens high in cholic acid require additional dilution.

1 cc. of the alcoholic solution of bile is measured into a test-tube (18 mm.), and most of the alcohol is evaporated by heating in a boiling water bath. Generally 7 to 8 minutes are sufficient for the evaporation of the alcohol. About 0.05 cc. of alcohol (enough to moisten the bottom of the tube) should remain or a small loss of taurocholic acid will result. When the tubes have cooled, the evaporated bile is dissolved in 2 cc. of freshly diluted 0.45 per cent (by volume) aqueous furfural solution, and 6 cc. of 16 N  $\text{H}_2\text{SO}_4$  are added. The remainder of the procedure is the same as that described for dog bile except that in this instance 1 cc. of an alcoholic solution of cholic acid (0.204 mg. per cc.) or the equivalent amount of sodium cholate serves as the standard. The alcohol is removed by evaporation as described above. Because of the insolubility of cholic acid in water, more uniform results are obtained if the  $\text{H}_2\text{SO}_4$  is added to it before the furfural.

Solutions of human bile sometimes become turbid during the heating with the furfural- $\text{H}_2\text{SO}_4$  reagent. When this occurs, 7 cc. of 95 per cent ethyl alcohol are added to both unknown and standard after the tubes have been cooled. After mixing thoroughly, the tubes are allowed to stand for a few minutes until bubbles no longer form before being read in the colorimeter. In rare instances this quantity of alcohol is not enough and a second portion is needed. If the material is pathological human bile, time is saved by preparing duplicate standards, one of which may be diluted with alcohol if required and the other used undiluted. Two precautions must be observed in comparing the alcoholic solutions colorimetrically. The plungers and bottoms of the cups of the colorimeter must be kept free from the minute bubbles that often form in these solutions, and when the unknown is more concentrated than the standard, the readings should be corrected for deviation from Beer's law. This deviation is practically linear, and only two points need to be established, in addition to that representing the standard, usually at  $1\frac{1}{2}$  and 2 times the concen-

tration of the standard. These are plotted against colorimeter readings in the usual manner (see Yoe, 1928), and the corrected values read directly from the curve. In the absence of alcohol, a linear relationship exists between the color formed and the concentration of cholic acid until the readings become difficult because of the depth of color; hence, correction is not required. The results are most satisfactory when the quantity of cholic acid in the unknown is equal to or less than that in the standard if no final dilution with alcohol is made. If alcohol is required to remove turbidity, it is advisable to compensate for the diminished color either by increasing the depth of the solutions read in the colorimeter, or by repeating the experiment with double the original quantities of unknown and standard.

In the analysis of bile, special care is needed in measurement by pipette owing to the high viscosity of many specimens. It is advisable to use pipettes calibrated to contain, and after delivering the bile, to rinse the pipette with the diluting fluid several times. The color of the specimen can generally be used as a guide to the dilution, although exceptions are frequently encountered. Because of the great variations in the composition of bile it is safest to start with comparatively low dilutions.

The sulfuric acid reagent should be standardized by titration. We have encountered one lot of sulfuric acid which was not suitable for use, since it gave a purple tint instead of a clear dark blue with furfural and cholic acid. The blank for this acid was high, and the total color formed by cholic acid was diminished by one-half.

A filtered neon light similar to that described by Gregory and Pascoe was used as a source of illumination for all of the work reported in this paper; however, we have found that equally good results are obtained when an ordinary colorimeter lamp (with the blue daylight filter removed) is used with suitable filters. A red glass disc filter of the type supplied by several manufacturers for use in the colorimeter eyepiece is most convenient, although any type of filter having a sharp cut-off in the 6200 Å. region and a high transmission in the red would be suitable. The spectral transmission of our neon lamp and filters was essentially the same as that reported by Gregory and Pascoe, extending from 6150 Å. to about 6700 Å. with the greatest transmission in the region of 6400 Å.



## EXPERIMENTAL

The hue and intensity of the color formed in the reaction depend chiefly on the four factors, time and temperature of heating, and concentrations of  $\text{H}_2\text{SO}_4$  and furfural. In testing these variables each was examined in a series of combinations of the other three in order to establish the conditions yielding the greatest amount of color compatible with the practical application of the method.

To evaluate color formation at each concentration of acid properly, it was necessary first to find the optimum time of heating for each normality studied. With the aid of this information various concentrations of  $\text{H}_2\text{SO}_4$  were compared, extending from 7.5 N to 16.8 N. As the concentration is increased, the absorption of the effective wave-lengths rises rapidly to a plateau between 10 and 12 N and then falls sharply. A second rise occurs with about 14 N acid, caused for the most part by reaction between the  $\text{H}_2\text{SO}_4$  and furfural, and when the blank given by the reagents is subtracted, the color due to cholic acid is much less than at lower acidities. Because it gave maximal color at a comparatively rapid rate with a negligible blank, 12 N  $\text{H}_2\text{SO}_4$  was selected as the best concentration.

Although slightly more color is developed in shorter periods of heating at higher temperatures than  $70^\circ$ , fading occurs more rapidly and the decreased stability of the color more than offsets a possible gain. Besides, if the time is made too short (less than 5 minutes) by the use of higher temperatures, physical factors such as small variations in the diameter of the tubes become significant. The higher concentration of acid and slightly higher temperature make possible the decrease in the time from 30 minutes as recommended by Gregory and Pascoe to 8 minutes. There is little change in the color between 7.5 and 8.5 minutes, but above or below these limits the quantity of color decreases rapidly. If the higher limit is exceeded or if the tubes are overheated, the fading of the color is accompanied by the development of turbidity. The data for time and temperature may be applied directly only if 18 mm. test-tubes are used, although the general relationships are independent of this factor.

Additional furfural heightens the color until the concentration reaches 1.3 mg. per cc. This is about 3 times the amount used in the original method. At higher concentrations the color is

again diminished, while at the same time the blank given by the reagents increases markedly. The blank is under all conditions dependent to a considerable extent upon the state of the furfural, which should be colorless or yellow rather than brown. The blank is small under the conditions selected, but it will rise parallel with the development of brown color in the furfural. When stored in a refrigerator, furfural keeps sufficiently well for several months. Commercial redistilled c. p. furfural gave satisfactory results without further treatment, although samples that had been

TABLE I  
*Recovery of Glycocholic Acid Added to Bile*

Cholic acid in bile solution	Cholic acid added	Total cholic acid taken	Total cholic acid found
0.5 cc. alcoholic extract of hepatic bile of dog, diluted 83.3 times			
mg.	mg.	mg.	mg.
0.040	0.022	0.062	0.065
	0.043	0.083	0.083
	0.086	0.126	0.121
	0.129	0.169	0.164
	0.172	0.212	0.205
	0.215	0.255	0.252
0.5 cc. alcoholic extract of gallbladder bile of dog, diluted 250 times			
0.114	0.021	0.135	0.136
	0.042	0.156	0.159
	0.084	0.198	0.194
	0.126	0.240	0.227
	0.168	0.282	0.286
	0.211	0.325	0.303

kept in stock for some time were unsatisfactory. Completely eliminating the blank given by the reagents by lowering the concentration of furfural and  $\text{H}_2\text{SO}_4$  greatly diminishes the sensitivity of the method.

Representative results obtained with the modified technique are shown in Table I.

*Color Equivalence of Conjugated Cholic Acids*—One would expect about the same amount of color per mol from the conjugated acids as from cholic acid if the color-producing reaction depends

on the cholic acid nucleus. It is essential to know if this is true because of the possible existence in bile of unconjugated cholic acid, and also because of the desirability of using cholic acid or sodium cholate as standards (at present these seem to be the only suitable preparations commercially available in pure form). Taurocholic and glycocholic acids were purified according to the method of Hammarsten (1925). Total nitrogen determinations gave for two crystalline preparations of taurocholic acid 2.75 and 2.81 per cent (Samples 3 and 4 respectively). Theoretical is 2.71 per cent. The proportion of bound solvent was determined by heating a portion of each preparation at 100° for 24 hours; and the data for all preparations are corrected for loss of weight under these conditions. The decomposition of taurocholic acid which occurred, shown by the formation of a brown color, was disregarded in calculating the loss of weight. The melting points, specific rotation, and nitrogen contents of two preparations of glycocholic acid were determined. Preparation 1 had the following properties: m.p. 127° corrected,  $[\alpha]_D^{20} = 33.4^\circ$ , N = 3.07 per cent; Preparation 2: m.p. 124–128°,  $[\alpha]_D^{20} = 32.2^\circ$ . Others have found melting points 124–140°,  $[\alpha]_D^{20} = 32.3^\circ$ , N = 3.01 per cent. The cholic acid used was Schuchardt's *Purissima*. Its melting point was 196.6° corrected, compared with 196–197° given in the literature, and the  $[\alpha]_D^{20} = 36.9^\circ$ , which agrees well with 37.0°, the figure most frequently cited by other workers. These values did not change appreciably after two recrystallizations from alcohol.

The pure conjugated acids yielded as much color as an equimolar quantity of cholic acid. In terms of per cent of the calculated amount of cholic acid, the two preparations of glycocholic acid gave as an average of six determinations, 98.2 and 99.4 respectively. For taurocholic acid the corresponding figures for the three samples were 97.1, 99.6, and 95.0 per cent. However, this relationship exists only when the acids are treated with aqueous  $H_2SO_4$ . In the presence of more than 0.1 cc. of alcohol, the conjugated acids gave less color than cholic acid, the values decreasing as the amount of alcohol was increased. It was thought at first that this was caused by a process similar to that involved in the formation of choleic acids. Apparently this is not the explanation, for a number of substances known to form choleic acids did not affect the color formed from conjugated acids.

Desoxycholic acid is present in the bile of many species, and it is important to know if it will contribute to the color developed by cholic acid. We were not able to recover a sufficient quantity of the desoxycholic acids of human bile to make purification worth attempting, but through the courtesy of Dr. Carl H. Greene of the Mayo Clinic we were able to compare with cholic acid three samples of desoxycholic acid. The purest of these was a crystalline preparation of the sodium salt from Professor Wieland, and it gave no color. A sample of desoxycholic acetic acid purified by Dr. Greene gave only 7 per cent of the color which an equimolar amount of cholic acid would yield, while the third sample, desoxycholic acetic acid, prepared commercially, gave 15 per cent. It seems probable that this color was due to cholic acid not completely removed during the separation of the desoxycholic acid. Dehydrocholic acid gives a yellow color that is eliminated by the red filter. As desoxycholic acid does not yield color, it is unlikely that lithocholic acid, containing one hydroxyl group less, will do so, and it is probable, therefore, that the method is specific for the cholic acid series. One remaining possible reactant of importance in the analysis of human bile is anthropodesoxycholic acid which has not been tested.

*Behavior of Other Constituents of Bile*—The following substances neither increased nor inhibited the color developed from cholic acid: cholesterol, ergosterol, cholesteryl oleate, cholesteryl acetate, oleic acid, egg lecithin, tributyrin, bilirubin (crystalline), creatinine, arginine, cystine, glycine, histidine, proline, taurine, tyrosine, adenine nucleotide, uric acid, gelatin, and inositol. Only tryptophane and proteins containing it, phenol, and biliverdin gave color enough to require further consideration. Interfering proteins are removed by alcohol and heat at an early stage in the procedure. Neither tryptophane nor phenol is likely to be present in bile in significant amounts. The biliverdin tested by us yielded about 20 per cent as much color as an equal weight of cholic acid; however, some bile acid was probably present as an impurity. Biliverdin is not formed from bilirubin to any visible extent when the latter is heated with the reagents. Biliverdin is seldom present in sufficient amount in human bile to cause interference. It is frequently present in dog bile, judging from the green color of many specimens, although we have not encountered any in which

the error from this source was significant. It is advisable, of course, to start the analysis as soon as possible after collecting the bile, since in many specimens the oxidation of bilirubin begins as soon as exposure to air has occurred. This change can be retarded by protecting the specimen from oxygen, by storing in the dark and at low temperatures, or by diluting with alcohol.

It is noteworthy that two preparations of cholesteryl oleate gave only a faint pink color, and that barium oleate behaved in like manner. The cholesteryl oleate was synthesized by Mr. H. F. Snider from cholesterol and oleic acid by the method of Hürthle (1895-96). Walker (1930) has recently concluded that it is this substance in blood rather than bile acid which is responsible for the positive Pettenkofer reaction given by alcoholic extracts of blood. This is a sound objection to those methods depending upon comparisons of red or lavender tints, but the use of red monochromatic light removes any possibility of error from this source in the present method since the color given by cholesterol oleate does not absorb light in the wave-lengths used.

A few determinations have been made on the blood of hospital patients with normal serum bilirubin content. After removal of proteins and a portion of the fatty material, alcoholic extracts evaporated nearly to dryness were treated according to the improved procedure. In every case, solutions which absorbed the monochromatic red light were formed, although it was difficult to identify a blue color owing to the development of yellow or buff tints in the solutions. Assuming that this light absorption originated with cholic acid, there was present in the blood of these individuals less than 1 mg. per cent of cholic acid.

#### SUMMARY

By establishing optimal conditions in respect to the four principal variables, the concentrations of  $H_2SO_4$  and furfural, time and temperature of heating, the sensitivity of the Gregory and Pascoe method for the determination of bile acids was more than doubled.

A comparison of pure conjugated and unconjugated cholic acids showed that equimolar quantities produced equal amounts of color. Cholic acid was found to be best adapted for use as a standard. The reaction appears to be specific for the cholic acid series.

Various substances that might interfere with the application of the method were tested. Only biliverdin has possible significance, and then only under exceptional circumstances. Cholesteryl oleate produced a pink color which did not absorb light of the wave-lengths used; therefore, it is not the substance in blood giving the positive reaction for cholic acid.

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## OUABAIN OR *g*-STROPHANTHIN

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Ouabain was discovered and so named in 1888 by Arnaud<sup>1</sup> who isolated the crystalline glucoside from the bark and roots of the ouabaio tree, a species of *Acocanthera* used by the Somalis of East Africa as an arrow-poison. Soon afterward<sup>2</sup> he found the same glucoside in another arrow-poison, the inée or onaye of the Pahouins, which was prepared from the seeds of *Strophanthus gratus* (*Strophanthus glabre*, du Gabon). The name *g*-strophanthin was later introduced by Thoms<sup>3</sup> to distinguish it from the strophanthins of other species, such as *hispidus* (*h*-strophanthin) and *kombe* (*k*-strophanthin).

On the basis of the analysis of the glucoside, which crystallizes with 9 mols of water, and that of its barium salt Arnaud derived a formula,  $C_{30}H_{46}O_{12}$ ,<sup>1</sup> which has been generally adopted. However, the average of the analytical results published by him (C 59.70, H 7.65) which were obtained with the glucoside dried at 140° is really in closer agreement with a formula  $C_{29}H_{44}O_{12}$ . At the time he discussed the difficulty of weighing the hygroscopic dried material and later<sup>2</sup> based his conclusions on results obtained with a monohydrate which resulted on drying ouabain at 100°. Our own analyses of ouabain agree with the  $C_{29}$  formula. Other analyses reported by Thoms<sup>3</sup> and more recently by others<sup>4</sup> cannot be regarded as conclusive. We shall give below other reasons for the adoption of the formula  $C_{29}H_{44}O_{12}$ . By hydrolysis with acid

<sup>1</sup> Arnaud, M., *Compt. rend. Acad.*, **107**, 1011 (1888).

<sup>2</sup> Arnaud, M., *Compt. rend. Acad.*, **107**, 1162 (1888).

<sup>3</sup> Thoms, H., *Ber. pharm. Ges.*, **14**, 114 (1904).

<sup>4</sup> Schwartze, E. W., Hann, R. M., and Keenan, G. L., *J. Pharmacol. and Exp. Therap.*, **36**, 481 (1929).

Arnaud determined the presence of 1 mol of rhamnose.<sup>5</sup> The hypothetical aglucone,  $C_{23}H_{34}O_8$  ( $C_{24}H_{36}O_8$  of Arnaud), however, could not be obtained. Instead, non-crystalline resins were formed which analysis showed to be dehydration products.

Other extensive studies were made by Arnaud, the most important of which was the formation of a crystalline substance correctly diagnosed as an anhydroheptacetate when anhydrous ouabain was heated with acetic anhydride and  $ZnCl_2$ .<sup>6</sup> Although his explanation of the production of this substance was faulty in minor points, its formation definitely showed the function of the oxygen atoms of the aglucone, as we shall discuss below.

In the course of our studies on the structure of and interrelationships among the cardiac glucosides it was shown<sup>7</sup> that ouabain is an unsaturated lactone, since, like the other members of this group, it gives the nitroprusside reaction characteristic of  $\Delta^{\beta,\gamma}$  unsaturated lactones. On catalytic hydrogenation it yielded a dihydro derivative which no longer gave this reaction. This fact placed it at once in close relationship to the strophanthidin group of glucosides. Since strophanthidin and related aglucones have been shown to be  $C_{23}$  derivatives it appeared probable that the aglucone of ouabain possesses the formula  $C_{23}H_{34}O_8$  rather than that of  $C_{24}H_{36}O_8$  required by Arnaud's formula for ouabain of  $C_{30}H_{46}O_{12}$ . Ouabain itself should, therefore, be  $C_{29}H_{44}O_{12}$ . This has been established by our more recent work.

On continuing the study of ouabain repeated attempts have been made to prepare the aglucone by many modifications of the conditions of hydrolysis. Our experience, however, has been a repetition of that of Arnaud—only non-crystalline resins could be obtained, which analysis showed to result from dehydration accompanied perhaps by polymerization. A condition is found here which is not encountered in the case of cymarín, gitoxin, and similar glucosides in which the aglucones are joined to  $\alpha$ -desoxy sugars. It has been the common experience that such glucosides are very readily and completely hydrolyzed under very gentle conditions. In the case, however, of digitalinum verum the

<sup>5</sup> Arnaud, M., *Compt. rend. Acad.*, **126**, 346, 1208 (1898).

<sup>6</sup> Arnaud, M., *Compt. rend. Acad.*, **126**, 1654 (1898).

<sup>7</sup> Jacobs, W. A., and Hoffmann, A., *J. Biol. Chem.*, **67**, 333 (1926); **74**, 787 (1927).



aglucone, probably gitoxigenin, is joined to digitalose, a sugar in which the  $\alpha$ -carbon atom bears a hydroxyl group. Here again more severe hydrolytic conditions have been found necessary so that not gitoxigenin but its dianhydro derivative, digitaligenin, resulted.<sup>8</sup> Similarly, ouabain is a relatively stable rhamnose glucoside and the aglucone does not withstand the severe conditions required for its hydrolytic cleavage. Attempts to circumvent this difficulty by the use of enzymes have also proved futile.

Finally, with the hope of preventing the dehydration which accompanied hydrolysis we turned to a derivative of ouabain in which the hydroxyl groups are protected by acylation, namely, the anhydroouabain heptacetate of Arnaud. Aside from a revision of the formula to the  $C_{29}$  basis and slight differences in physical properties the original observations of Arnaud on this substance were confirmed. In addition we have shown the presence in it of two double bonds by catalytic hydrogenation which produced a tetrahydro derivative or *desoxydihydroouabain heptacetate*. Since ouabain already possesses a double bond the second double bond in the anhydroheptacetate must have been formed by loss of 1 mol of water during the acetylation. The tetrahydro derivative was then submitted to hydrolytic studies. After futile attempts by the usual methods a successful result was obtained by the method of acetolysis, but with unexpected complications. When the hydrogenated acetate was heated with an acetic acid-hydrochloric acid mixture a crystalline cleavage product was obtained. On analysis figures were obtained which agreed closely with a formula  $C_{24}H_{30}O_4$ . Repeated analyses of numerous preparations gave the same result. On saponification with alkali 2 equivalents were consumed, due to the cleavage respectively of a lactone group and of an acetyl group. On reacidification the free *hydroxylactone*,  $C_{22}H_{28}O_3$ , was obtained. Further study afforded an explanation of the origin and nature of the above acetolysis product. On the basis of the formula  $C_{23}H_{34}O_8$  for the ouabain aglucone and therefore of the formula  $C_{23}H_{36}O_7$  for its desoxydihydro derivative, a pentacetate of the latter should normally be expected upon acetolysis of the above desoxydihydroouabain heptacetate. One of the acetyl groups of this pentacetate would cover the hydroxyl group

<sup>8</sup> Windaus, A., and Schwarte, G., *Ber. chem. Ges.*, **58**, 1515 (1925).

formerly attached to the sugar. Since instead of such a pentacetate a monoacetate was actually isolated, the latter must have been formed by the subsequent loss of 4 acetic acid molecules from the pentacetate with the production of four double bonds. In the latter case, such a monoacetate should have a formula  $C_{25}H_{30}O_4$ . Since the formula actually determined was  $C_{24}H_{30}O_4$ , it was necessary to account for the additional complication of this loss of 1 carbon atom. The clue to the difficulty was found in the fact that during the acetolysis formaldehyde was formed as a cleavage product. This was shown to originate in the aglucone portion of the molecule, since rhamnose itself under the conditions of the acetolysis produced no formaldehyde. It is conceivable that in the tetraanhydro derivative of the aglucone presumably formed

as an intermediate there is a grouping  $\begin{array}{c} \text{C} \\ \diagup \quad \diagdown \\ \text{C} = \text{CH}_2 \\ \diagdown \quad \diagup \\ \text{C} \end{array}$  which is

cleaved to  $\begin{array}{c} \text{C} \\ \diagup \quad \diagdown \\ \text{CH}_2 \\ \diagdown \quad \diagup \\ \text{C} \end{array}$  and  $\text{CH}_2\text{O}$ . This is suggested by the well

known cleavage of pulegone by acids into acetone and methylcyclohexanone.

In accordance with this reaction the intermediate tetra unsaturated acetate,  $C_{25}H_{30}O_4$ , would lose 1 carbon atom and one double bond during the cleavage of formaldehyde and a *triply unsaturated monoacetate*,  $C_{24}H_{30}O_4$ , would result. This was substantiated by the fact that the acetate absorbed 3 mols of catalytically activated hydrogen with the formation of at least two isomeric *saturated acetates*,  $C_{24}H_{34}O_4$ , which were readily separated. The less soluble isomer preponderated. On saponification these isomers gave two isomeric ( $\alpha$ - and  $\beta$ -) *saturated hydroxylactones*,  $C_{22}H_{34}O_3$ . The hydroxyl group of these substances proved to be of secondary character. On oxidation of the  $\alpha$  isomer it yielded a beautifully crystallizing *ketolactone*,  $C_{22}H_{32}O_3$ .

In analogy to our experience with other cardiac aglucones the inference is permissible that this secondary hydroxyl group which emerges as the acetate from the above acetolysis was the point of attachment of the sugar on the aglucone. Furthermore, the

four hydroxyl groups which are acetylated in Arnaud's anhydroheptacetate and then removed during acetolysis are probably of tertiary character. Finally, the particular hydroxyl group which is removed in the formation of Arnaud's anhydroheptacetate is probably the tertiary hydroxyl group corresponding to  $\text{OH}^1$  of the strophanthidin group of aglucones. The presence of such a group was substantiated by the formation of *isououabain* when anhydrous ouabain was treated with methyl alcoholic alkali by the usual method for the preparation of isoaglucones. The resulting isououabain no longer gives the Legal reaction.

From these results, ouabain is a rhamnose glucoside of a hexahydroxytetracyclic- $\Delta^{\beta,\gamma}$  unsaturated lactone,  $\text{C}_{23}\text{H}_{34}\text{O}_8$ , of the strophanthidin group.

Unfortunately, the further correlation of this substance with strophanthidin is made difficult for the moment by the fact that the aglucone derivative of ouabain contains 1 less carbon atom. An attempt is being made to accomplish this correlation by a similar preliminary degradation of an appropriate strophanthidin derivative.

#### EXPERIMENTAL

*Anhydroouabain Heptacetate*—This substance was prepared according to the directions given by Arnaud.<sup>1</sup> The physical properties of the substance as obtained by us differed somewhat from those given by Arnaud. After repeated recrystallization, it melted at  $283\text{--}285^\circ$  and showed the rotation  $[\alpha]_D^{21} = -59.1^\circ$  ( $c = 1.01$  in pyridine) and  $[\alpha]_D^{21} = -51.7^\circ$  ( $c = 0.532$  in 85 per cent alcohol). Arnaud gave the melting point of  $310^\circ$  and the rotation as  $[\alpha]_D = -68.6^\circ$  ( $c = 0.730$  in 85 per cent alcohol).

The crystalline form of our substance and its solubility relationships agreed with those described by Arnaud.

3.897 mg. substance:	2.333 mg. $\text{H}_2\text{O}$ ,	8.565 mg. $\text{CO}_2$
3.945 "	" : 2.365 "	8.670 " "
	$\text{C}_{23}\text{H}_{34}\text{O}_{11}$ . Calculated.	C 59.97, H 6.55
	Found.	" 59.94, " 6.70
		" 59.94, " 6.71

*Dihydrodesoxyouabain Heptacetate*—20 gm. of carefully recrystallized anhydroouabain heptacetate were suspended in 300 cc. of 95

per cent alcohol. The mixture was treated with 0.5 gm. of the platinum oxide catalyst of Adams and Shriner and shaken with hydrogen. The hydrogenation was usually ended within 24 hours after the absorption of approximately 2 mols of  $H_2$ . The sparingly soluble hydrogenation product gradually replaced the starting material. At this point the reaction product was dissolved by warming, and after addition of decolorizing carbon to coagulate the catalyst, the solution was filtered. The filtrate on cooling yielded the hydrogenation product. Concentration of the mother liquor to about 100 cc. gave a second crop. After recrystallization from 85 per cent alcohol, it formed colorless, irregular, very thin leaflets which melted at  $273-275^\circ$ . The yield was 13 to 17 gm. On further concentration of the mother liquors, lower melting material was recovered, possibly consisting of isomers, the separation of which was not attempted.

$$[\alpha]_D^{25} = -63.8^\circ \text{ (c = 0.980 in pyridine)}$$

4.122 mg. substance: 2.590 mg.  $H_2O$ , 9.055 mg.  $CO_2$

3.895 " " : 2.345 " " 8.568 " "

3.830 " " : 2.350 " " 8.430 " "

$C_{43}H_{60}O_{18}$ . Calculated. C 59.69, H 7.00

Found. " 59.91, " 7.03

" 59.99, " 6.73

" 60.03, " 6.86

12.857 mg. of substance were refluxed in a mixture of 1 cc. of alcohol and 3 cc. of 0.1 N NaOH for 5 hours and then titrated back against phenolphthalein. Calculated for 8 equivalents (one lactone and seven acetyl groups), 1.190 cc. Found, 1.219 cc.

When hydrogenation was performed in acetic acid solution, the yield of the above substance was lower.

*Acetolysis of Dihydrodesoxyouabain Heptacetate.* Trianhydrolactone Monoacetate,  $C_{24}H_{30}O_4$ —10 gm. of heptacetyldihydrodesoxyouabain were dissolved in 50 cc. of glacial acetic acid containing 3 per cent of dry hydrogen chloride. This solution was heated in a sealed tube for 40 minutes at  $100^\circ$ . A shorter period of heating diminished the yield considerably; a longer time did not increase the yield, but favored the formation of tarry by-products which were difficult to separate from the desired product. The reaction mixture was poured slowly into 400 cc. of water contain-

ing an excess of sodium acetate. The resulting amorphous precipitate was collected with water. After solution of the dried substance in 95 per cent alcohol, crystallization slowly occurred. The yield was 1.3 gm., or about 30 per cent of the theory. After two recrystallizations from alcohol the substance formed fairly large, usually truncated pyramids with hexagonal bases, which melted at 172–173°.

The following analytical results are representative of numerous determinations made on a large number of different preparations.

$$[\alpha]_D^{25} = -108.4^\circ \text{ (c = 1.08 in pyridine)}$$

3.805 mg. substance; 2.692 mg.  $H_2O$ , 10.540 mg.  $CO_2$

$C_{24}H_{10}O_4$ . Calculated. C 75.36, H 7.90

Found. " 75.55, " 7.92

A molecular weight determination according to the method of Rast gave the following results.

21 508 mg. camphor; 2.173 mg. substance,  $\Delta = 10.8$  ( $K = 43.0$ ). Mol. wt., calculated, 380.2; found, 402.0.

12.670 mg. of substance were refluxed with 1 cc. of alcohol and 3 cc. of 0.1 N NaOH for 4.5 hours and then titrated back with phenolphthalein. Calculated for 2 equivalents, 0.667 cc.; found, 0.706 cc.

This substance was also isolated in the form of long, exceedingly slender needles by solution in the minimum amount of chloroform, followed by dilution of this solvent with ethyl alcohol. Occasionally these needles crystallized out simultaneously with the *hexagonal based* pyramids. The needles had the same melting point and optical rotation as the pyramids and could be converted into the latter form by recrystallization from boiling alcohol. Apparently the two forms represent polymorphic modifications of the same substance. When the substance was recrystallized from a sufficiently large volume of ether, the two forms were again encountered.

The trianhydroacetate is readily soluble in acetone, chloroform, pyridine, acetic acid, and hot alcohol; it is sparingly soluble in ether and the cold alcohols. The substance gives a bright yellow coloration when dissolved in tetranitromethane.

It was possible to demonstrate the presence of formaldehyde in the mother liquors from the precipitation of the crude acetolysis product as follows: These mother liquors were acidified with 25 cc. of sulfuric acid and then distilled slowly through a long condenser. The distillate and vapors were collected under ice water. Dilute ammonia was added to the distillate from time to time, in quantity sufficient to keep the solution faintly alkaline. About 150 cc. were collected. This distillate gave the positive ring test for formaldehyde with resorcinol and sulfuric acid. This test was given even with subsequent samples of distillate. The ammoniacal distillate after heating for a few minutes was concentrated under diminished pressure to 20 cc. On addition of iodine-sodium iodide solution, a crystalline precipitate formed which was identified by color, crystalline form, and melting point as hexamethylenetetramine tetraiodide. The yield was 0.1 gm.

A repetition of this procedure with more distillate from the acetolysis mother liquors gave additional hexamethylenetetramine tetraiodide.

As a check, 2 gm. of rhamnose were subjected to acetolysis. Considerable decomposition and charring took place. The resorcinol ring test for formaldehyde on the distillate was negative and no hexamethylenetetramine tetraiodide could be isolated from the ammonia condensate.

*Trianhydrohydroxylactone*,  $C_{22}H_{28}O_8$ —A solution of 0.5 gm. of the above acetate in 5 cc. of ethyl alcohol and 5.5 cc. of *N* sodium hydroxide was refluxed for 3.5 hours. The reaction mixture was then diluted with 10 cc. of water, cooled to room temperature, and made acid to Congo red with 10 per cent hydrochloric acid. A colorless amorphous resin separated. The mixture was allowed to stand overnight for relactonization and was then extracted with chloroform. The concentrated extract yielded on addition of ether long needles which melted at 196–197°. After recrystallization from methyl alcohol it melted at 198–199°.

$[\alpha]_D^{20} = -41.5^\circ$  ( $c = 0.94$  in pyridine)

4.502 mg. substance: 3.300 mg.  $H_2O$ , 12.860 mg.  $CO_2$

4.225 " " : 3.140 " " 12.075 " "

$C_{22}H_{28}O_8$ . Calculated. C 77.58, H 8.29

Found. " 77.90, " 8.20

" 77.94, " 8.32

14.260 mg. of substance were refluxed with 1 cc. of alcohol and 3.0 cc. of 0.1 N NaOH for 3.5 hours and then titrated back against phenolphthalein. Calculated for 1 equivalent, 0.419; found, 0.449 cc.

*$\alpha$ -Hexahydrolactone Monoacetate,  $C_{24}H_{38}O_4$* —A solution of 0.88 gm. of the trianhydrolactone acetate in 50 cc. of acetic acid was hydrogenated in the presence of 0.5 gm. of platinum oxide catalyst which had previously been reduced. The absorption of hydrogen proceeded steadily, and after 8 hours practically stopped when 3.15 mols of hydrogen had been absorbed. During the next 14 hours no evidence of further absorption was obtained. After removal of the catalyst the clear solution was concentrated under diminished pressure to small volume. On dilution with water the crystalline substance deposited. This proved to be a mixture of at least two isomers which were separated by fractionation from 95 per cent alcohol. After repeated recrystallization the less soluble substance which preponderated weighed 0.33 gm. It formed long, irregular prisms which melted at 203–204°. It was readily soluble in acetone and chloroform and sparingly soluble in ether and methyl and ethyl alcohols. The substance gave no coloration when dissolved in tetranitromethane.

$$[\alpha]_D^{20} = -22.2^\circ \text{ (c = 1.03 in pyridine)}$$

4.058 mg. substance: 3.310 mg.  $H_2O$ , 11.047 mg.  $CO_2$

4.418 " " : 3.685 " " 12.070 " "

3.800 " " : 3.220 " " 10.407 " "

$C_{24}H_{38}O_4$ . Calculated. C 74.17, H 9.35

Found. (a) " 74.24, " 9.13

(b) " 74.51, " 9.33

(c) " 74.68, " 9.48

*$\beta$ -Hexahydrolactone Monoacetate*—The more soluble isomer was found in the mother liquors from the above crystallizations. It was finally obtained from the concentrated alcoholic solution and after repeated recrystallization formed long compact prisms which melted at 167–169°. The yield was 70 mg.

$$[\alpha]_D^{20} = -21.5^\circ \text{ (c = 1.03 in pyridine)}$$

4.095 mg. substance: 3.410 mg.  $H_2O$ , 11.210 mg.  $CO_2$

4.425 " " : 3.740 " " 12.075 " "

$C_{24}H_{38}O_4$ . Calculated. C 74.17, H 9.35

Found. " 74.66, " 9.32

" 74.42, " 9.46

*$\alpha$ -Hexahydrohydroxylactone,  $C_{22}H_{34}O_3$* —The above  $\alpha$ -acetate was saponified in the usual manner. On reacidification to Congo red with dilute hydrochloric acid a colorless resin separated. This was allowed to stand overnight for relactonization. The suspension was then extracted with ether. The ethereal solution left a residue which was dissolved in a small volume of methyl alcohol. On cautious dilution colorless needles crystallized. After two recrystallizations from dilute methyl alcohol the substance melted at 94–96°.

$[\alpha]_D^{25} = -8.9^\circ$  ( $c = 1.13$  in ethyl alcohol)

4.002 mg. substance: 3.495 mg.  $H_2O$ , 11.180 mg.  $CO_2$

3.673 " " : 3.190 " " 10.253 " "

$C_{22}H_{34}O_3$ . Calculated. C 76.25, H 9.88

Found. " 76.19, " 9.77

" 76.13, " 9.72

*$\beta$ -Hexahydrohydroxylactone,  $C_{22}H_{34}O_3$* —This substance was prepared by saponification of the  $\beta$ -acetate and was isolated as in the case of the  $\alpha$  isomer. It formed colorless needles from dilute methyl alcohol which melted at 164–166°.

$[\alpha]_D^{25} = +11.2^\circ$  ( $c = 1.08$  in ethyl alcohol)

4.273 mg. substance: 3.840 mg.  $H_2O$ , 11.935 mg.  $CO_2$

3.863 " " : 3.505 " " 10.783 " "

$C_{22}H_{34}O_3$ . Calculated. C 76.25, H 9.88

Found. " 76.18, " 10.06

" 76.13, " 10.15

*$\alpha$ -Hexahydroketolactone,  $C_{22}H_{32}O_3$* —A solution of 30 mg. of the  $\alpha$ -hexahydrohydroxylactone in 1 cc. of 90 per cent acetic acid was treated with 0.08 cc. of Kiliani's solution. After 10 minutes the reaction mixture was diluted. The ketone crystallized at once. After recrystallization from dilute acetone, it formed irregular leaflets which melted at 188–191°.

4.185 mg. substance: 3.495 mg.  $H_2O$ , 11.778 mg.  $CO_2$

3.645 " " : 3.070 " " 10.265 " "

$C_{22}H_{32}O_3$ . Calculated. C 76.69, H 9.37

Found. " 76.76, " 9.34

" 76.80, " 9.42

*Isoouabain*—5 gm. of anhydrous ouabain were dissolved in 25 cc. of absolute methyl alcohol. The solution was chilled to 10°.



Occasionally the ouabain crystallized at this point. To the cold solution or suspension were added 25 cc. of a 20 per cent solution of potassium hydroxide in absolute methyl alcohol. The solution was allowed to stand at room temperature, protected from moisture, for 1 hour. The clear solution was poured, with stirring, into 4 times its volume of a mixture of ice and water. Before the ice had melted, the solution was carefully acidified to Congo red with strong hydrochloric acid and then allowed to stand overnight for relactonization. The acidity of the solution was then carefully adjusted until neutral to Congo red, but still acid to litmus. After concentration to dryness under diminished pressure the residue was extracted with boiling absolute alcohol. The combined alcoholic extracts were filtered and concentrated to dryness. The resulting syrup was dissolved in 15 cc. of hot water, followed by 10 cc. as rinsings. This concentrated solution on standing in the refrigerator, especially after seeding, deposited a hydrate of isouabain in clusters of small needles. The product was collected and washed with a small quantity of chilled water. This material showed a tendency to sinter together. The yield was 1.7 gm. On recrystallization from a small volume of water either needles or irregular leaflets separated, which softened at about 200° and melted with slow effervescence at 213–215°. Further recrystallization did not affect the melting point or optical rotation.

Isoouabain crystallizes as a hydrate, the exact composition of which was not determined. The water is lost on drying at 115° *in vacuo*. The following rotation was determined on the substance dried at room temperature over calcium chloride.

$$[\alpha]_D^{20} = -45.7^\circ \quad (c = 1.05 \text{ in water})$$

Isoouabain is readily soluble in methyl and ethyl alcohols, fairly readily soluble in water and acetone, and practically insoluble in ether, petroleum ether, chloroform, and benzene. It does not give the Legal reaction.

For analysis the substance was dried at 115° and 15 mm.

3.980 mg. substance: 2.750 mg. H<sub>2</sub>O, 8.695 mg. CO<sub>2</sub>

3.847 " " : 2.640 " " 8.427 " "

C<sub>29</sub>H<sub>44</sub>O<sub>12</sub>. Calculated. C 59.56, H 7.58

Found. " 59.58, " 7.73

" 59.74, " 7.68

*Ouabain*—The following analyses were made with samples of ouabain which had been recrystallized from water at least six times and kept in a desiccator over  $\text{CaCl}_2$ .

The substance was dried for analysis at  $115^\circ$  and 20 mm.

3.804 mg. substance: 2.620 mg.  $\text{H}_2\text{O}$ , 8.318 mg.  $\text{CO}_2$

3.965 “ “ : 2.755 “ “ 8.670 “ “

4.169 “ “ : 2.860 “ “ 9.120 “ “

$\text{C}_{29}\text{H}_{44}\text{O}_{12}$ . Calculated. C 59.56, H 7.58

Found. “ 59.64, “ 7.71

“ 59.64, “ 7.77

“ 59.66, “ 7.68

## A NEW TRIPLE ACETATE METHOD FOR SODIUM DETERMINATIONS IN BIOLOGICAL MATERIALS

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It has been known for years that sodium combines with zinc uranyl acetate or magnesium uranyl acetate in a definite ratio to form yellow crystals, soluble in water but only slightly soluble in alcohol. In 1886, Streng (1) studied this compound and employed it for the detection of sodium. He ascribed to it the formula  $(\text{UO}_2)_3\text{MgNa}(\text{CH}_3\text{COO})_9 \cdot 9\text{H}_2\text{O}$ . According to Miholic (2) the compound contains only 6 molecules of water. While Blanchetière (3) corroborates the statement of Streng, the findings of Barber and Kolthoff (4) agree with those of Miholic. Barber and Kolthoff state that the ratio of the weight of sodium to the weight of the triple acetate  $(\text{UO}_2)_3\text{ZnNa}(\text{CH}_3\text{COO})_9 \cdot 6\text{H}_2\text{O}$  is 0.01495:1. Furthermore, Barber and Kolthoff (4, 5), who were the first to employ this compound as a basis for quantitative estimation of sodium, made a thorough study of the influences of various ions on sodium estimations by this method. They state that barium, calcium, magnesium, and ammonium do not interfere; cesium and rubidium likewise do not interfere if present in quantities below 0.1 gm. Lithium, strontium, and organic acids, as oxalic and tartaric acids, do interfere. Potassium interferes if present in large amounts; *i.e.*, 50 mg. per 1 cc. Phosphates and arsenates interfere, but may be removed easily with magnesia mixture.

The reagents employed by these investigators in the quantitative estimation of sodium were (a) uranyl zinc acetate saturated with uranyl zinc sodium acetate, and (b) 95 per cent alcohol saturated with this triple acetate. Sodium was precipitated by the addition of 20 cc. of uranyl zinc acetate to every 2 cc. of the sample. The precipitate was transferred to a glass filter crucible

on a suction flask where it was first washed with the 95 per cent alcohol saturated with triple acetate and then with ether. The precipitate was dried to constant weight, and the sodium was estimated by multiplying the total weight by 0.01495.

The results published by Barber and Kolthoff show that the method was satisfactory when applied to samples containing relatively large amounts of sodium; *i.e.*, 75 to 494 mg.

Butler and Tuthill (6) applied a slightly modified Barber-Kolthoff method to urine, and obtained satisfactory results with samples containing as little as 1.5 mg. of sodium. For the removal of phosphates powdered calcium hydroxide was employed. The method was satisfactory for blood serum, but at least 1 cc. was necessary and it had to be digested with concentrated sulfuric acid.

In my experience, the Barber-Kolthoff method gives satisfactory results with quantities employed by Butler and Tuthill, but cannot be satisfactorily used with smaller quantities. Caley (7) tried the method on samples containing 0.1 to 0.5 mg. of sodium with unsatisfactory results.

McCance and Shipp (8) devised a colorimetric method in which the color was developed with potassium ferrocyanide. They obtained satisfactory results with even smaller quantities of sodium than those used by Caley. In the hands of the writer, however, their method failed to give consistent results. Several other workers have experienced difficulties with the method.

From a study of various modifications of the original Barber-Kolthoff method it is apparent that the principal difficulty with this method lies in the solubility of triple acetate in water. Even if the zinc uranyl acetate reagent is saturated with the triple acetate, an addition of 2 cc. of a sample containing about 0.1 mg. of sodium to 10 cc. of the reagent reduces the concentration of the triple acetate to such an extent that none of the sodium comes down as a precipitate. Precipitation in the cold and the use of large amounts of the reagent are the only known means for overcoming this difficulty. However, if the temperature and time are not strictly controlled, refrigeration is of no value. If the temperature is too low, not only the sodium of the sample is precipitated but some of the reagent and practically all the triple acetate with which it is saturated. Furthermore, if the experi-

ment is performed in warm weather, some of the precipitate goes into solution during centrifuging and draining.

In our laboratory, where it is desirable to make accurate determinations of sodium in a drop of aqueous or vitreous humor, without being able to run duplicates, a more reliable method is necessary. This has been accomplished by a method in which sodium is precipitated quantitatively from the uranyl zinc acetate reagent, without saturation with triple acetate, at room temperature by the use of alcohol. Furthermore, by a series of experiments, it was found that glacial acetic acid saturated with triple acetate is a far better wash reagent than alcohol saturated with triple acetate. The entire determination is made at room temperature and in detail is as follows:

*Major Reagents and Appliances*

1. Uranyl zinc acetate reagent as prepared by Butler and Tut-hill (6) but without saturation with triple acetate. Solution A consists of 80 gm. of sodium-free uranyl acetate (c.p., Baker's Analyzed) and 46 cc. of 30 per cent acetic acid (by volume) plus water to make 520 gm. Solution B consists of 220 gm. of zinc acetate and 23 cc. of 30 per cent acetic acid plus water to make 520 gm. The solutions are made by placing the ingredients in covered beakers and heating over a steam bath with occasional stirring until dissolved. The two solutions are mixed while hot, allowed to stand for 24 hours, then filtered into a bottle. Shortly before using, the necessary quantity is removed with a pipette and again filtered. The excess is replaced in the bottle.

2. Absolute alcohol or 95 per cent alcohol.

3. Glacial acetic acid (c.p., Baker's Analyzed, special, 99.6 to 99.7 per cent) saturated with sodium uranyl zinc acetate. Approximately 1 gm. of triple acetate is placed in a bottle, moistened with the least possible quantity of distilled water, 1 liter of glacial acetic acid is added, and the contents are well mixed. Before use, the bottle is well shaken and the precipitate allowed to settle. The necessary quantity is then filtered into a large test-tube.

4. A 20 per cent aqueous solution of potassium ferrocyanide (c.p., Baker's Analyzed). It is filtered if necessary.

5. Standard sodium chloride solution. Exactly 5 gm. of sodium

chloride (C.P., Baker's Analyzed) are dissolved in a 500 cc. volumetric flask and made up to volume. 1 cc. of this solution contains 10 mg. of sodium chloride or 3.9345 mg. of sodium.

6. Whatman No. 42 filter paper is used in all filtrations.

7. Stirring rods: No. 1, a fine glass rod about 0.5 mm. in diameter; No. 2, a larger glass rod about 1 mm. in diameter. Each of these is made by heating glass tubing and pulling out into a fine rod; the ends are sealed and flattened.

*Minor Reagents (for Sodium Determinations in Biological Materials)*

1. Powdered calcium hydroxide (Baker's Purified). This contains traces of sodium; however, the same quantity is added to both sample and standard and does not affect the end result.

2. A 20 per cent trichloroacetic acid solution (U.S.P., Baker's or Merck). It is filtered after 24 hours.

3. Concentrated ammonium hydroxide (sp. gr. 0.9, C.P., Baker's Analyzed).

4. A 1 per cent solution of phenolphthalein in absolute alcohol.

5. Perchloric acid (sp. gr. 1.54, Baker's Analyzed).

*General Procedure*

Exactly 2 cc. of the sample solution (containing 0.05 to 0.5 mg. of sodium) are delivered into a 15 cc. centrifuge tube. The same quantity of a standard sodium chloride solution, containing approximately the same amount of sodium, is measured into another centrifuge tube of the same size. To each are added 6 cc. of freshly filtered uranyl zinc acetate reagent. With a 1 cc. Mohr pipette graduated into 0.1 cc. exactly 0.3 cc. of absolute alcohol is added to each tube. The alcohol is stirred into the solution with the No. 1 stirring rod. The bulk of the precipitate is allowed to settle into the lower half of the solution, then another 0.3 cc. of alcohol is added to each tube and the stirring repeated without disturbing the precipitate on the bottom. The same stirring rod is used for each tube, without rinsing. After a few minutes another 0.3 cc. of alcohol is added and stirred again. This procedure is repeated four more times, seven times in all, until 2.1 cc. of alcohol have been added. The time intervals are shortened after the second or third addition; however, the process of precipitation consumes at least  $\frac{1}{2}$  hour. (If the entire quantity of

alcohol is added at once, the results are not satisfactory.) When precipitation is complete, the contents of the tubes are centrifuged for 10 minutes. The supernatant fluid is decanted and the tubes, still held in an inverted position, are placed on coarse filter paper and allowed to drain. The precipitate is washed with 5 cc. of freshly filtered saturated solution of triple acetate in glacial acetic acid. The reagent is delivered from a pipette by gentle blowing; the pipette is pressed against the wall of the tube immediately below its edge while the tube is rotated between the fingers. The precipitate is thoroughly stirred with a No. 2 stirring rod and distributed uniformly throughout the wash reagent. Before transferring the stirring rod to the next tube, it is rinsed with a few drops of wash reagent. After centrifuging, the tubes are drained as before, the mouth of each tube is then wiped with a moist cloth to remove all traces of uranyl zinc acetate reagent, and to each tube is added a drop of glacial acetic acid. The walls of the tubes are rinsed with distilled water from a wash bottle until the solution reaches a point 1 inch below the top. The precipitate is dissolved and evenly distributed throughout the tube by thorough stirring. The contents of the tubes are transferred by means of a small funnel to 50 cc. volumetric flasks. The tubes are filled with water as before; the contents are stirred and transferred to the volumetric flasks. This procedure is repeated once again, and finally the funnel is rinsed, and the volume made up to mark. The contents of each flask are well mixed by shaking. If the amount of sodium lies between 0.1 and 0.2 mg. and the standard contains approximately the same amount, exactly 20 cc. are pipetted from each flask into large test-tubes.<sup>1</sup> To each tube is added 0.5 cc. of 20 per cent potassium ferrocyanide solution and the contents are well mixed. (The color should never be developed before making up the solution to the desired volume.) After standing 5 minutes, the samples are ready for reading in the colorimeter. If one works with amounts of sodium between 0.2 and 0.3 mg., only 10 cc. of the contents of the volumetric flasks are pipetted into the test-tubes and the volumes are made up to 20 cc. by adding to each tube 10 cc. of distilled water followed by thorough mixing with a stirring rod. When one is working with uncertain quanti-

<sup>1</sup> Test-tubes, 6 × 1 inch, are preferable.

ties of sodium, the concentration of sodium (or uranium) in each sample must be ascertained by trial experiments. 2 or 3 cc. of each solution are placed in small test-tubes and a drop of potassium ferrocyanide solution is added. From this a rough estimate may be made as to the extent each should be diluted before developing the color. Thus the contents of one tube may be made up of 5 cc. of distilled water and 15 cc. of sample solution, another may be made up of equal amounts of distilled water and sample solution, while a third one may need no dilution. If the final volumes are the same, the dilutions of samples and standard are expressed in the calculation as follows:

$$\frac{15_a \times 0.39345 \times 10}{16.4 \times 0.1 \times 15_b} = \text{mg. Na per 1 cc. urine}$$

where  $15_a$  is the reading of the standard; 16.4, the reading of the sample; 0.39345, mg. of sodium in the standard; 0.1, cc. of urine in sample; 10, cc. of standard solution contained in the 20 cc.;  $15_b$ , cc. of the sample solution contained in the 20 cc.

If many sodium determinations are made simultaneously, color should never be developed in more than six samples at the same time. For a new series of samples another portion of the standard solution contained in the 50 cc. volumetric flask is used and in this the color is developed. Since the intensity of the color undergoes a gradual change, it is well to make fewer readings than to make many readings at the expense of time. A rise in temperature accelerates this color change, consequently the light in the illuminating chamber should be turned off during preparation of the next sample.

#### *Application of Method to Biological Materials*

*Blood Serum*—To 0.5 cc. of serum diluted to 5 cc. in a 15 cc. centrifuge tube are added 5 cc. of 20 per cent trichloroacetic acid solution. At first the acid must be added drop by drop under vigorous stirring, then more rapidly. After standing a few minutes the tube is centrifuged. Exactly 2 cc. of the supernatant fluid are placed in another 15 cc. centrifuge tube. At the same time the standard is prepared as follows: 5 cc. of standard sodium chloride stock solution (1 cc. of which equals 3.9345 mg. of sodium) are diluted to 100 cc. in a volumetric flask with 10 per cent trichloro-



acetic acid solution, and 2 cc. (0.39345 mg. of Na) are placed in a 15 cc. centrifuge tube. To the sample and the standard are added 6 cc. of freshly filtered uranyl zinc acetate reagent and the treatment is continued as outlined under "General procedure." Since the sample contains more than 0.3 mg. of sodium, only 10 cc. of sample and 10 cc. of standard solution are pipetted into the test-tubes; they are made up to 20 cc. by adding 10 cc. of distilled water before developing the color.

#### *Calculation*

$$\frac{R \text{ standard} \times 0.39345 \times 100}{R \text{ sample} \times 0.1} \quad \text{mg. Na per 100 cc. serum}$$

Each experiment usually consisted of two determinations carried out as follows: The blood serum was divided into two parts. After adding to one a known quantity of standard sodium chloride solution and precipitating the protein in both by trichloroacetic acid, 2 cc. of each were employed. The results are given in Table I.

*Whole Blood (from Finger Tip)*—In this procedure only 0.2 cc. of blood is necessary. This amount of blood is collected in a serological pipette and discharged into a 15 cc. centrifuge tube, containing exactly 3 cc. of distilled water. The protein is precipitated by the slow addition, with constant stirring, of 0.8 cc. of 20 per cent trichloroacetic acid. The tube is centrifuged, and 2 cc. of the supernatant fluid are used to obtain the precipitate as described under "General procedure." The data obtained are given in Table II.

*Urine*—Different urines vary widely in sodium content, and for this reason duplicate or triplicate analyses with varying amounts of urine are run. The bulk of phosphates, uric acid, etc., are first precipitated by allowing the urine to stand in a refrigerator for several hours. After filtering, 3 cc. of the filtrate are placed in a 15 cc. centrifuge tube containing about 0.3 gm. of dry calcium hydroxide. At the same time 3 cc. of a standard sodium chloride solution, prepared by diluting 15 cc. of the standard sodium chloride stock solution to 100 cc. and containing 0.5902 mg. of Na per cc., are pipetted into a similar tube containing 0.3 gm. of calcium hydroxide. The volumes of the standard and sample are made up to 10 cc. by adding to each tube 7 cc. of 1

TABLE I  
*Blood Serum*

Subject No.	Experiment No.	Na in serum	Na calculated	Na found	Recovery
		<i>mg. per cent</i>	<i>mg.</i>	<i>mg.</i>	<i>per cent</i>
1	1	359.9	0.2780	0.2784	100.0
1	2	359.9	0.2783	0.2797	100.4
1	3	357.7	0.2772	0.2720	98.1
1	4*	359.9	0.2783	0.2771	99.6
1	5*	353.2	0.2750	0.2745	99.8
1	6*	353.2	0.2750	0.2720	98.9
2	1	355.5	0.3745	0.3745	100.1
3	1	347.1	0.4629	0.4582	99.0
3	2	347.1	0.4629	0.4611	99.6

\* These experiments differed from others in the following details: After the protein was precipitated in both the sample and the sample plus the standard with trichloroacetic acid in a 0.5:5 ratio, 2 cc. of the supernatant fluid, in a 15 cc. centrifuge tube, were neutralized with 2 cc. of 3 per cent ammonium hydroxide. A small quantity of powdered calcium hydroxide was then added to each sample, including the standard. After allowing the tubes to stand overnight, they were centrifuged, and 2 cc. of the supernatant fluid were used in the precipitation of the sodium uranyl zinc acetate. The results are slightly lower, and the difference may be attributed to the removal of phosphates. It is evident that there is no necessity for the removal of phosphates.

TABLE II  
*Whole Blood*

Subject No.	Experiment No.	Na per 100 cc. blood
		<i>mg.</i>
1	1	194.1
2	1	198.1
2	2	199.4
2	3	196.7
2	4	200.7
3	1	209.3
3	2	209.3

per cent ammonium hydroxide, and the contents are thoroughly mixed with a small stirring rod. The tubes are allowed to stand at room temperature for at least an hour, during which time the contents are repeatedly stirred. If the tubes stand overnight,

they should be closed with rubber stoppers. After centrifuging, the supernatant fluid of the sample is prepared in triplicate as follows: (1) 1 cc. of the fluid is diluted to 4 cc. with distilled water. This is well mixed, and 2 cc. (0.15 cc. of urine) are pipetted into a 15 cc. centrifuge tube. (2) 1 cc. of the fluid (0.3 cc. of urine) is diluted with distilled water to 2 cc. in a 15 cc. centrifuge tube. (3) 2 cc. of the fluid (0.6 cc. of urine) are pipetted into a third 15 cc. centrifuge tube. All three tubes and one standard containing 2 cc. of the supernatant fluid are then carried through the general procedure at the same time. By comparing the amounts of precipitate after the addition of the uranyl zinc acetate reagent and

TABLE III  
*Urine*

Sample No.	Experiment No.	Urine used	Na per 100 cc. urine	Na in sample	Na in standard	Na calculated	Na found	Recovery
		cc.	mg.	mg.	mg.	mg.	mg.	per cent
1	1	0.12	119.2	0.1431	0.3148	0.2289	0.2248	98.2
2	1	0.12	322.5	0.3225	0.3148	0.3509	0.3500	100.0
3	1	0.20	172.3	0.3446	0.3148	0.3297	0.3302	100.1
4	1	0.20	83.7	0.0877	0.3148	0.2076	0.2018	97.2
5	1	0.60	64.7	0.3883	0.3935	0.3343	0.3430	102.6
5	2	0.60	66.0	0.3961	0.3935	0.3388	0.3392	100.1
6	1	0.60	60.7	0.3643	0.3935	0.3206	0.3208	100.0
6	2	0.60	61.1	0.3666	0.3935	0.3219	0.3208	99.7

alcohol, the one approximating the standard is selected. If a duplicate is desired, the two closest to the standard are retained.

If the urine contains much protein, the latter is removed by adding sufficient solid trichloroacetic acid to make a 10 per cent solution. After thorough shaking, the specimen is filtered and the filtrate is neutralized to phenolphthalein with ammonium hydroxide and made up to such a volume that 10 cc. are equivalent to 3 cc. of urine. This is pipetted into a 15 cc. centrifuge tube containing the necessary amount of dry calcium hydroxide for the removal of phosphates.

The data in Table III show the recoveries when determinations were run in pairs, with one sample consisting of urine only, and the other of urine and a known quantity of standard sodium chloride.

*Tissue*—Fresh beef muscle was used in all experiments. About 10 or 15 gm. of tissue are placed in a porcelain mortar and covered with 3 cc. of 20 per cent trichloroacetic acid. It is carefully macerated with the pestle until all protein has completely coagulated, after which 10 cc. of distilled water are added, and the maceration continued. The mortar is then tilted with the lip downward and the coagulated mass pushed upward and pressed as dry as possible. With the solid mass held in place with the pestle, the extract is filtered into a 50 cc. beaker. The residue is further macerated with 2 cc. of trichloroacetic acid solution and 8 cc. of water. After the second extract is filtered into the beaker, 1 cc. of trichloroacetic acid is placed in the mortar, the pestle and mortar are rinsed with a few cc. of distilled water and maceration is continued. After this extract is filtered into the beaker, final extraction is made with a few cc. of distilled water. When the final extract has filtered through, the filter is washed three times with 2 cc. of distilled water. In another 50 cc. beaker are placed 2 cc. of standard sodium chloride stock solution (3.9345 mg. of Na per cc.) and 6 cc. of 20 per cent trichloroacetic acid solution, also sufficient distilled water to make a total volume of approximately that of the extract. The contents of the beakers are evaporated on a steam bath to approximately 15 cc. and neutralized to phenolphthalein with concentrated ammonium hydroxide. To each beaker is added 0.5 gm. of powdered calcium hydroxide. The contents are well stirred and digestion is continued for  $\frac{1}{2}$  hour, after which the beakers are cooled and allowed to stand at room temperature for 1 or more hours. The contents are filtered into 50 cc. volumetric flasks and the filters are washed as previously described. 2 cc. of both the sample and the standard are used in the precipitation of the triple acetate.

In working with larger quantities of tissue, *e.g.* 20 or 30 gm., proportionately larger quantities of trichloroacetic acid and calcium hydroxide are used, and the final volumes are made up to 100 cc.

*Calculation*—If the standard contains 0.3148 mg. of sodium, and the final volume of the extract from exactly 10 gm. of tissue is 50 cc., the following formula is employed.

$$\frac{R \text{ standard} \times 0.3148 \times 100}{R \text{ sample} \times 0.4} = \text{mg. Na per 100 gm. tissue}$$

In five experiments, a different sample of beef muscle being used each time, pairs of determinations were run as follows: The tissue was divided into two unequal parts. To the smaller part a known quantity of standard sodium chloride solution was added in the mortar before maceration, and both samples were carried through the process in exactly the same way. The results are listed in Table IV.

Attempts were made to destroy the organic matter in tissue by dry ashing. Both nickel and vitreosol crucibles were employed, but the results were unsatisfactory. With new crucibles the results were too low, whereas after the crucibles were used the results were too high. The explanation suggested is that in new crucibles

TABLE IV  
*Tissue*

Sample No.	Experiment No.	Tissue, large sample	Na, large sample	Na, 100 gm tissue	Na in standard	Na calculated	Na found	Recovery
		<i>gm.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>per cent</i>
1	1	0.3114	0.1741	55.9	0.3148	0.2544	0.2559	100.6
2	1	0.3119	0.1760	56.5	0.3148	0.2809	0.2810	100.0
3	1	0.2784	0.1927	69.2	0.3148	0.3300	0.3300	100.0
4	1	0.2879	0.1828	63.5	0.3148	0.3403	0.3472	102.0
5	1	0.7281	0.4536	62.3	0.3148	0.6203	0.6212	100.2
5	2	0.7281	0.4718	64.8	0.3148	0.6294	0.6212	98.7

some of the sodium fuses with the nickel or vitreosol and tends to accumulate in the crucibles.

*Stool*—Ashing of stools for sodium determinations is rejected for the above reasons. A procedure therefore was devised by which sodium is extracted with dilute alcohol. After the sample is thoroughly mixed in a beaker, it is placed in a short stemmed funnel. This is held over a tared 100 cc. lipless cylinder on a torsion balance, and, with the aid of a stirring rod, exactly 20 gm. of the material are delivered through the funnel into the cylinder. The cylinder is filled with 95 per cent alcohol to mark 90 cc., closed with a rubber stopper, and shaken until a more or less homogeneous mass is obtained. After the addition of 1 cc. of concentrated ammonium hydroxide, the volume is made up with distilled water to 100 cc. In another similar cylinder, containing 1 cc.

of concentrated ammonium hydroxide and 70 cc. of 95 per cent alcohol, are placed exactly 3 cc. of standard sodium chloride stock solution (3.9345 mg. of Na per cc.) and the volume made up to 100 cc. with distilled water. To each cylinder are added 2 gm. of powdered calcium hydroxide and the cylinders are stoppered and shaken. They are allowed to stand overnight at room temperature, the shaking of the sample being repeated at frequent intervals during the evening. The supernatant fluid of the sample is filtered into a small reagent bottle, then the entire contents of the cylinder are brought on the filter paper and covered with a watch-glass. Exactly 20 cc. of filtrate and standard are pipetted into 25 cc. volumetric flasks and evaporated to dryness in an electric oven at a temperature between 80–100°. Second portions of 20 cc. of each are pipetted into the same flasks and again evaporated to dryness. To each flask are added 2 cc. of perchloric acid and a drop of concentrated sulfuric acid; the contents are then digested over a low flame, an asbestos pad being used as a support. The digests are cooled, dissolved in 10 cc. of distilled water, and neutralized to phenolphthalein with concentrated ammonium hydroxide. The volumes are made up to mark and 2 cc. of each are employed as outlined in "General procedure."

*Calculation*—If a total of 40 cc. of the filtrate is evaporated, the final quantity (2 cc.) of the sample will represent  $\frac{20 \times 40 \times 2}{100 \times 25} = 0.64$  gm. of stool. The final quantity of standard will contain

$$\frac{(3 \times 3.9345) \times 40 \times 2}{100 \times 25} = 0.3777 \text{ mg. Na}$$

Therefore, if the dilutions are the same for both the standard and sample, the final result, after the colorimetric readings, is

$$\frac{R \text{ standard} \times 0.3777}{R \text{ sample} \times 0.64} = \text{mg. Na per 1 gm. stool}$$

Two different samples of stools were analyzed. Each consisted of a pair of determinations, one of which was carried out on the sample only (20 gm. of stool), the other on the sample plus a known quantity of standard (20 gm. of stool plus 4 cc. of standard sodium chloride stock solution). In the latter case only half the amount

of filtrate specified above was evaporated for digestion. The results are given in Table V.

*Ocular Humors*—If the source is the human eye, from which slightly more than 0.2 cc. of aqueous humor can be obtained, exactly 0.2 cc. of the fluid is placed in a small test-tube and made up to 4 cc. with distilled water. It is well mixed with a stirring rod and 2 cc. are employed as outlined under "General procedure." In working with animal eyes, larger quantities of fluid may be employed and diluted proportionately so that 2 cc. of the solution represent 0.1 cc. of the aqueous humor. Before diluting the vitreous humor in a similar fashion, the protein is precipitated by adding a few crystals of trichloroacetic acid to 10 cc. of vitreous humor and stirring until all is dissolved. After being filtered, it

TABLE V  
*Stools*

Experi- ment No.	Stool used	Na per 100 gm. stool	Na in sample	Na in standard	Na calcu- lated	Na found	Recovery
	gm.	mg.	mg.	mg.	mg.	mg.	per cent
1	0.44	65.1	0.2264	0.3148	0.1725	0.1689	97.9
2	0.64	44.3	0.2835	0.3148	0.2678	0.2684	100.1

is treated like the aqueous humor. The standard is prepared as described for serum.

Results on ocular humors will be published later.

#### SUMMARY

A new triple acetate method for sodium is presented in which the sodium is precipitated with alcohol in a 15 cc. centrifuge tube, centrifuged, and washed with glacial acetic acid saturated with the triple salt. The sodium in the precipitate is estimated either gravimetrically or colorimetrically, depending on the amount of precipitate. The optimum quantity of sodium for the colorimetric procedure is approximately 0.3 mg. However, satisfactory results, with an experimental error of only 2 or 3 per cent, may be obtained with as little as 0.05 mg. of sodium.

The method is adapted, without resort to dry ashing, to sodium estimations in biological materials such as serum, whole blood, urine, tissue, stools, and ocular humors.

My thanks are due to Dr. C. S. O'Brien, Head of the Department of Ophthalmology, for his valuable help and suggestions throughout the work.

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## **STUDIES IN THE PHYSIOLOGY OF VITAMINS**

### **XIX. THE ACID-BASE BALANCE OF THE BLOOD DURING LACK OF UNDIFFERENTIATED VITAMIN B\***

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(Received for publication, March 28, 1932)

A careful survey of the literature fails to reveal any studies of the acid-base balance of the blood during lack of the vitamin B complex. From previous investigations conducted in this laboratory it was strongly suggested that dogs in the advanced stages of undifferentiated vitamin B deficiency have an acidosis. Stucky and Rose (1) observed a progressive increase in blood concentration and the secretion of a highly concentrated urine during the development of the syndrome leading eventually to polyneuritis; this was associated with a voluntary decrease in fluid consumption. Rose (2) demonstrated that the convulsions manifested by a polyneuritic animal could be temporarily relieved by injections of Ringer-Locke solution. Although the favorable response to such therapy was transitory, this treatment, nevertheless, "appeared

\* The physiological properties of what was formerly called vitamin B have since been shown to be due to at least two substances which have been designated vitamins B and G by some investigators, and B<sub>1</sub> and B<sub>2</sub> by others. The terms undifferentiated vitamin B and vitamin B complex used in this paper refer to the mixture of these two substances present in the product used as a source of vitamin.

Part of the data in this paper are taken from a dissertation presented by Ethel Burack in partial fulfillment of the requirements for the degree of Doctor of Philosophy, Yale University, June, 1931.

The expenses of this research were defrayed in part by grants from the Committee on Therapeutic Research, Council on Pharmacy and Chemistry, American Medical Association, and the Research Fund at Yale University School of Medicine.

† Alpha Xi Delta Fellow of the American Association of University Women for the academic year 1930-31, and Standard Brands, Incorporated, Fellow, 1931-32.

to be of distinct value in enabling the animal to tide over a critical period." Administration of physiological saline is a common clinical method for correcting the blood electrolyte pattern (3). It would seem, therefore, that the transitory efficacy of Ringer-Locke injections reported by Rose (2) could be explained by a retention of fluid and electrolytes necessary for the restoration of the normal alkaline reserve.

In investigating such a problem it is important to take account of the inanition which occurs in animals deprived of the vitamin B complex (4). It has been shown by Gamble, Ross, and Tisdall (5) that fasting results in a loss of water from the body, which is accompanied by a loss of salts, so that osmotic equilibrium of the fluids is maintained. Because of the exaggerated protein catabolism during prolonged fasting, the increased excretion of acid radicals causes the removal of base, and thus water, so that acidosis has a dehydrating effect. Tissue dehydration is in most cases encountered in conjunction with acidosis (6). It is possible, therefore, that if the acid-base balance in dogs suffering from advanced vitamin B avitaminosis is abnormal, the self-imposed fasting, which occurs during the subjection of animals to a vitamin B-deficient regimen, may be the responsible factor. Hence it seemed profitable to study the acid-base equilibrium of the blood during deficiency of undifferentiated vitamin B, giving special attention to the control of food and water fasting which complicates this condition.

#### EXPERIMENTAL

Eighteen dogs were used for these studies. During a preliminary period observations were made of their readiness to eat the experimental ration, Casein III (7), the daily caloric requirement for the maintenance of body weight, and the volume of water voluntarily consumed each day.

Three pairs of animals were used in the first series of experiments. One member of each pair was given water *ad libitum* and that amount of the basal diet which was found to satisfy the caloric needs of the animal. The daily intakes of food and water were measured. The other member of the pair served as the control and was limited, proportionately, to the amount of food and water that was consumed by the experimental companion the

previous day. As a source of antineuritic vitamin B the controls received daily 1 gm. of Vitavose<sup>1</sup> per kilo of body weight.

In the second series the procedure was slightly modified. Six of the eight animals were placed in two groups of three each. Two dogs of each group received the deficient ration and the third animal served as the control. The latter was given the *least amount* of food and water consumed by either of the other members so that secondary changes obtained as a result of inanition would be represented in the most aggravated form. Observations of blood lactic acid were made on the four remaining dogs.

### *Methods*

Blood was withdrawn under oil from the jugular vein when the animal was in the postabsorptive condition. The serum was obtained for analysis by the procedure outlined by Austin, Cullen, Hastings, McLean, Peters, and Van Slyke (8). Carbon dioxide was determined by the micro method of Van Slyke and Neill (9). For the estimation of chlorides the procedure of Van Slyke (10) with certain modifications was employed. Inorganic phosphate was determined by the method of Benedict and Theis (11). Measurement of serum protein was obtained by subtracting the non-protein nitrogen from the total nitrogen and multiplying by the factor 6.25. Total nitrogen was estimated by subjecting 0.4 cc. of serum to the macro-Kjeldahl procedure (12), and the non-protein nitrogen by the method of Folin and Wu (13). Total base was determined according to the method described by Stadie and Ross (14). This has been found to be accurate without correction for the phosphate (15).

For the lactic acid studies the blood was obtained from the femoral artery and was analyzed for lactic acid by the Friedemann and Kendall modification of the Friedemann, Cotonio, and Shaffer method (16).

The concentrations of the serum electrolytes were calculated in terms of milli-equivalents (17).

### *Results*

In the first series of animals, which represented three vitamin B-deficient dogs and three companion controls, all the principal

<sup>1</sup> Vitavose is a wheat germ preparation made by E. R. Squibb and Sons, New York.

factors concerned in the acid-base balance of the blood were determined; namely, bicarbonate, chloride, protein, inorganic phosphate, and total base. Although complete analyses were made *once every week* from the beginning of the regimen until onset of the symptoms, due to lack of space, only those data are tabulated here which either reveal a change in electrolyte composition or represent a significant day in the experiment.

*Electrolyte Composition of Normal Canine Serum*—The normal ranges of concentrations of total base and principal acids of dog serum were found to be the same as those characteristic for human serum, with the exception of the chloride concentration, as Table I indicates.

*Blood Electrolyte Changes during Lack of Undifferentiated Vitamin B*—Illustrative data are presented in Table II. Dogs 1, 3, and 5

TABLE I  
*Normal Ranges of Serum Electrolyte Concentrations*

Constituent	Human serum	Dog serum
	m.-eq.	m.-eq.
Chloride.....	97 -105	108 -112
Bicarbonate .....	21 - 28	21 - 25
Protein.....	16 - 20	16 - 20
Inorganic phosphate .....	1.4- 2.6	1.7- 2.6
Total base.....	150 -160	150 -160

subsisted on the vitamin B-deficient diet. Dog 1 never fasted severely during the experiment. The appetite was erratic during the latter half of the period, but a complete and prolonged anorexia did not occur. As a result, there was only a moderate loss in body weight. Examination of the data obtained from this animal reveals no significant changes in the concentrations of the individual electrolytes during the entire period. It will be noticed that on the day when polyneuritis was manifested, the bicarbonate level fell slightly below normal. On the day following vitamin therapy, bicarbonate, chloride, and total base were found to have increased. Evidence will be furnished later to show that these changes can be accounted for by liberation of base bound by lactic acid which was produced by the muscular activity during the convulsions.

### Concentrations of Serum Electrolytes in Dogs Deficient in Vitamin B Complex and Their Controls

Dog No.	Day of experiment	Body weight	HCO <sub>3</sub>	Cl	Protein	Inorganic P	Total acid, (4) + (5) + (6) + (7)	Total base	Remarks
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
		kg.	m.-eq.	m.-eq.	m.-eq.	m.-eq.	m.-eq.	m.-eq.	
1, vitamin B-deficient		11.9	24.1	109	18.2	1.9	153.2	158.6	Appetite erratic Polyneuritis After vitamin therapy
	42	11.7	23.8	108	17.8	2.2	151.8	159.0	
	68	10.7	22.4	108	18.0	2.3	150.7	162.6	
	80	9.9	17.1	104	18.9	2.5	142.5	153.5	
2, control		10.6	23.5	109	18.1	1.8	152.4	160.5	
		15.0	22.4	112	17.6	1.7	153.7	159.6	
	68	14.0	22.4	111	16.5	1.9	151.8	160.0	
	80	13.6	21.2	108	16.1	1.9	147.2	160.0	
3, vitamin B-deficient		12.0	22.3	111	17.5	2.0	152.8	158.4	Partial anorexia. Fluid intake markedly diminished Polyneuritis 1 day after vitamin administration
	129	12.2	19.9	116	16.9	2.2	155.0	165.8	
		9.6	19.0	110	17.1	2.1	148.2		
	194		21.5	113	18.1	1.8	154.4		
4, control		13.0	21.2	112	19.7	1.7	154.6	159.3	
	129	13.6						164.8	
	194	12.1	21.7	106	18.8	2.5	149.0		
5, vitamin B-deficient		11.9	23.3	110	16.3	1.8	151.4	157.2	
	68	8.6	19.5	114	14.4	3.1	151.0	166.0	
	77	7.6	17.2	101	14.0	2.2	134.4	164.3	
	78	7.5	11.2	95	13.8	2.2	122.2	153.5	

TABLE II—*Concluded*

Dog No.	Day of experiment	Body weight	HCO <sub>3</sub>	Cl	Protein	Inorganic P	Total acid, (4) + (5) + (6) + (7)	Total base	Remarks
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
		kg.	m.-eq.	m.-eq.	m.-eq.	m.-eq.	m.-eq.	m.-eq.	
5—Continued		9 5	19.2	114	14.5	2.8	150 5	160 9	4 days after vitamin administration
		10.8	22.4	110	15.5	2.0	149.9	157.3	1 wk. after vitamin therapy
6, control		14.0	25.0	110	18 3	2 0	155 3	161.3	
	68	12 2	21.6	116	17.1	2.0	156 7	167 2	
	78	11.9	21.8	111	16.1	1 6	150.5	166 3	

Dog 3 was first observed to be a coprophagist after being on the experimental regimen for 120 days. Very shortly after the animal was muzzled the appetite became erratic and the fluid intake was markedly decreased. The high value for total base on the 129th day can be explained by the ingestion of salt with inadequate consumption of water, as the total base level of the companion control (Dog 4) concomitantly increased. It is known that marked increase in total base of the blood occurs in dogs after water deprivation (18). It is again noteworthy that, although the concentrations of the acid constituents did not vary outside of the normal limits, both the bicarbonate and chloride increased slightly on the day following vitamin therapy, suggesting the replacement of lactic acid that had been liberated the previous day when the polyneuritic symptoms were in evidence.

The history of Dog 5 illustrates the severe degree of inanition which may occur when the vitamin B complex is withheld. After 11 days of complete anorexia with very little water intake the serum protein level fell slightly and the total base was very high. The acid-base balance, after 21 days of complete fasting and markedly diminished water intake, showed a high concentration of undetermined acid. The bicarbonate and protein were slightly, and the chloride significantly, decreased, while the total base was elevated. On the following day the dog was in such an emaciated

state that it was impossible to obtain a blood sample by venous puncture. Although the results on serum obtained from arterial blood cannot be strictly compared with those from venous blood, due to differences in bicarbonate, chloride, and water content (19), they do show an accumulation of unknown acids similar in magnitude to that calculated from the analyses of the previous day (30 milli-equivalents). *There were no manifestations of convulsions.* After vitamin therapy and the restoration of appetite (note subsequent gains in weight) the blood electrolyte pattern became normal. That this condition is due in part to the food and water fast is demonstrated by the data obtained from the control animal, in which an increase in total base is seen to have occurred on the 68th and 78th days. In view of the fact that the acidosis exhibited by the vitamin B-deficient animal was much more severe than that of the companion receiving the vitamin, it may be argued that along with the changes due to inanition there are also accompanying changes referable specifically to vitamin starvation. On the other hand, it may be that the phenomenon is ultimately related to the inanition *per se*. From the body weights expressed in Table II it can be calculated that the vitamin B-deficient Dog 5 lost 38 per cent of its weight, as compared with only a 15 per cent loss suffered by the control (Dog 6). It is obvious, therefore, that a satisfactory statement as to the significance of the difference in electrolyte pattern is difficult to formulate.

In the second series of experiments further data were obtained in connection with another study, in which time did not permit determination of more than the electrolytes principally concerned in the regulation of body fluid reaction; namely, bicarbonate, chloride, and total base. From the previous study just reviewed it had already become evident that serum protein and inorganic phosphate vary only slightly during lack of undifferentiated vitamin B. This finding is confirmed by the study of Schelling (20), in which no significant changes in serum calcium, phosphorus, and protein were observed during vitamin B avitaminosis in dogs fed the Cowgill diet (7). Furthermore, as has been pointed out by Drake, Marsh, and Gamble (21), chloride and bicarbonate being the two chief acid ions of the blood, data of these two electrolytes together with total base "usually provide a nearly complete description of plasma changes accounting for a change in reaction."

**TABLE III**  
**Concentrations of Principal Electrolytes of Serum in Dogs Deficient in Vitamin B Complex and Their Controls**

Dog No.	Day of experiment	Body weight	HCO <sub>3</sub>	Cl	Total determined acid, (4) + (5)	Total base	Undetermined acid (E), (7) - (6)	Remarks
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
		kg.	m.-eq.	m.-eq.	m.-eq.	m.-eq.	m.-eq.	
10, vitamin B-deficient	10	8.8	22.4	113	135.4	159.4	24.0	Initial body weight, 8.9 kilos
	99	5.8	21.7	105	126.7	153.2	26.5	Polyneuritis
11, control	27	17.3	19.5	113	132.5	158.7	26.2	Initial body weight, 18 kilos
	100	13.0	20.7	110	130.7	153.4	22.7	
7, vitamin B-deficient	11	12.8	22.2	110	132.2	159.0	26.8	
	34	10.5	18.9	109	127.9	157.5	29.6	Partial anorexia
	39	9.9	23.5	102	125.5	158.5	33.0	Polyneuritis
9, vitamin B-deficient	11	17.7	22.2	107	129.2	156.0	26.8	Initial body weight, 18 kilos
	27	15.5	25.6	105	130.6	163.0	32.4	Complete anorexia 12 days
	52	12.4	19.1	104	123.1	160.2	37.1	Died of polyneuritis after 30 days almost complete anorexia
8, control	11	9.1	23.0	109	132.0	156.0	24.0	Initial body weight, 9.5 kilos
	27	7.7	22.7	104	126.7	162.4	35.7	12 days fasting
	53	5.8						Dog in critical condition after 30 days almost complete fasting
13, vitamin B-deficient	34	6.1	25.8	100	125.8	159.4	33.6	Initial body weight, 8.4 kilos. Anorexia 19 days
	51	5.2	19.8	100	119.8	155.0	35.2	Polyneuritis
			21.4	112	133.4	155.4	22.0	3 days after vitamin administration
15, vitamin B-deficient	34	12.0	22.4	105	127.4	156.4	29.0	Initial body weight, 13 kilos. Partial anorexia
	59	10.5	19.1	107	126.1	161.8	35.7	Polyneuritis
			22.4	106	128.4	154.2	25.8	1 day after vitamin and saline administration
14, control	34	11.0	22.6	104	126.6	156.7	30.1	Initial body weight, 13.8 kilos
	52	10.0	22.3	111	133.3	158.8	25.5	



The results obtained from this group of animals are given in Table III. It is seen that Dog 10 showed no abnormalities in acid-base balance even at the time of polyneuritic manifestations. In the case of the other animals lacking vitamin B (Dogs 7, 9, 13, and 15), it is noteworthy that, although the levels of the individual electrolytes at no time varied significantly outside of the normal range, there was nevertheless a progressive rise in undetermined acids up to the time of polyneuritis, and a subsequent fall in this fraction after vitamin therapy (see Dogs 13 and 15).

From the data yielded by the two control animals (Dogs 8 and 14) it cannot be determined definitely how much of the change is produced as a result of the inanition. One is here confronted by the difficulty of properly correlating the severity of fasting; *i.e.*, the extent of body weight loss, with the degree of change produced in the blood picture. Dog 8 fasted much more severely than Dog 14, in fact, more than any other control used in these studies. After 12 days of complete fasting, analysis of the blood showed a significant rise in the undetermined acid fraction (*R*). On the 53rd day the animal was found to have lost 40 per cent of its body weight and was consequently in such a critical condition that it was not possible to obtain a sufficient amount of blood for analysis. The clinical picture was similar in every way to that of Dog 5 discussed above, which reached such a state before onset of paralytic symptoms.

In considering the question as to what ions are responsible for the increase in the undetermined acid fraction (see Column 8, Table III), it is pertinent to call attention to the results obtained in the first series of experiments (Table II) and the work of Schelling (20), which furnish evidence that this rise is not due to changes in concentration of protein and inorganic phosphate. It has recently been reported that there is an increase in lactic acid of the blood (22), the brain (23), and heart, liver, and muscle (24) of birds exhibiting symptoms of polyneuritis. In view of these findings an attempt was made to determine the blood lactic acid in dogs fed and controlled according to the technique already described. The project was finally abandoned because the method of determining lactic acid did not always yield reliable results. However, from the study of two vitamin B-deficient animals and their respective controls (Table IV) it was evident that (1) there is no rise in lactic

acid during at least the first 3 weeks of the experimental period, (2) fasting does not have any effect upon the concentration of blood lactic acid in dogs, and (3) there is a marked rise in lactic acid during polyneuritis, which level returns to normal when the symptoms of the disease are removed.

The normal value of blood lactic acid expressed in milli-equivalents is approximately 1.5. At the time of polyneuritis these

TABLE IV  
*Concentrations of Lactic Acid and Sugar in Blood of Dogs Deficient in Vitamin B Complex, and Their Controls*

Day of experiment	Vitamin B-deficient animals			Controls		
	Body weight	Blood lactic acid	Blood sugar	Body weight	Blood lactic acid	Blood sugar
	kg.	mg per 100 cc.	mg. per 100 cc.	kg	mg. per 100 cc	mg. per 100 cc.
	9.4	10.4		8.5	8.6	
11	9.4	15.8		8.0		
18	8.9	13.2		7.7	6.6	
25	7.9	23.0		7.0	11.1	
35	7.4	20.2	75	6.8	18.6	77
54*	5.9	50.4	178	5.8	17.9	77
56†	5.8	18.8	109			
	10.7	13.7		10.5	12.4	
7	10.5	15.4	86	10.3	17.6	86
32	9.0	15.6	88	8.7	12.0	90
50*	7.6	47.4	115	6.8	13.5	90
52†	7.3	13.4	75			

\* Early stage of polyneuritis.

† The symptoms were removed by intravenous injection of a vitamin B concentrate kindly furnished by Dr. R. J. Block, but no food was given until *after* the blood sample was taken.

animals exhibited a concentration of lactic acid of  $\frac{50 \times 10}{90}$ , or about 5.5 milli-equivalents. The slight fall in the bicarbonate and chloride which was observed on the day of polyneuritic symptoms (Tables II and III) could therefore easily be accounted for by the increase in the lactic acid concentration which occurs at this time. It is obvious that the level of lactic acid in the blood will depend on the extent of the muscular activity during the mani-

festations of the symptoms. Thus, in the advanced stages of the disease an animal which exhibits frequent and severe convulsions will undoubtedly have a much higher concentration of lactic acid than the dogs here reported, which were given the vitamin early after the symptoms appeared. Values as high as 90 and 116 mg. per cent (10 to 13 milli-equivalents) were obtained by Barr and Himwich (25) in human subjects 3 minutes after *very severe* exercise.

The rise in undetermined acids which occurred in the two animals that suffered severe weight losses (Dogs 5 and 8) cannot be explained by the accumulation of lactic acid. In conditions of extreme fasting, other factors, such as individual susceptibility to acidosis, must come into play. Although it is a generally known fact that dogs ordinarily develop no more than trivial ketosis (if any) on fasting, idiosyncrasy and age are important factors to consider. In extensive studies of acidosis in dogs on fasting and high fat feeding, Allen (26) reported two dogs which evidenced definite ketosis. The interesting observation was also made that puppies are subject to marked ketosis when fasted even for short periods (27).

#### SUMMARY AND CONCLUSIONS

Studies of the acid-base equilibrium of the blood were made on dogs subsisting on a diet deficient in the vitamin B complex. The accompanying voluntary fasting, with respect to both food and water, was adequately controlled.

In animals which ate well during the experimental period no significant alterations in the concentrations of serum electrolytes were observed. The dogs which fasted exhibited changes believed to be related to the inanition of greater or lesser degree which accompanies deprivation of the vitamin B complex.

In general, the results of the investigation indicate that it is difficult to bring about a condition of acidosis in the dog either by simple fasting or by a lack of undifferentiated vitamin B. Shortage of this dietary factor is not primarily responsible for any characteristic changes in the acid-base balance of the blood.

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## STUDIES IN THE PHYSIOLOGY OF VITAMINS

### XX. THE GLUCOSE TOLERANCE DURING LACK OF UNDIFFERENTIATED VITAMIN B\*

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The question as to whether vitamin B plays a rôle in the normal metabolism of carbohydrate has long been debated. Funk and others (1, 2) have asserted that there is a relation between the amount of carbohydrate in a vitamin B-deficient ration and the time required for the development of avian polyneuritis; an increase in the carbohydrate portion of the diet is believed to hasten the onset of the symptoms. Vedder (3), however, repeated Funk's experiments, with results which failed to harmonize with this theory. In the most recent work on this subject Guha (4) could not demonstrate any relation between the period of onset of the disease and either the amount or the nature of the carbohydrate in the diet.

\* The physiological properties of what was formerly called *vitamin B* have since been shown to be due to at least two substances which have been designated vitamins B and G by some investigators, and vitamins B<sub>1</sub> and B<sub>2</sub> by others. The term *undifferentiated vitamin B* used in this paper refers to the mixture of these substances. The phenomenon studied in this investigation, however, was essentially due to deficiency of the antineuritic factor (vitamin B<sub>1</sub>).

Part of the data in this paper are taken from a dissertation presented by Ethel Burack in partial fulfilment of the requirements for the degree of Doctor of Philosophy, Yale University, 1931.

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† Alpha Xi Delta Fellow of the American Association of University Women, 1930-31, and Standard Brands, Incorporated, Fellow, 1931-32.

The blood sugar concentration of animals lacking vitamin B has also received considerable attention. Hyperglycemia in birds, dogs, and other species has been reported by several investigators (5-7). These workers, however, failed to control the factor of inanition which is involved during deprivation of vitamin B. When this was taken into consideration, it was found in the case of rats (8) and pigeons (9) that the changes in blood sugar level which were observed could be accounted for by the accompanying fasting. Bell (10) recently reported that "the incidence of hyperglycemia" in the pigeon "has been associated with the convulsive stage." This was not observed in the case of rats, which showed no change in blood sugar level even during the critical period. The results of Stucky and Rose (11) indicated that "vitamin B does not play a specific rôle in the regulation of the blood sugar level" in the dog.

In seeking to answer this question as to the relation of vitamin B to carbohydrate utilization several investigators turned to a study of the gaseous metabolism. Bickel (12) reported that the oxygen consumption and carbon dioxide production diminish during vitamin B avitaminosis, with a decrease in the respiratory quotient. He believed that there is an incomplete oxidation of sugar, with the production of toxic intermediate products. Magne and Simmonet (13) concur in this opinion. However, several workers have correlated the decline in heat production with the concomitant decrease in food consumption (14-16). Studies on dogs fed according to the Cowgill technique (17) were carried out by Deuel and Weiss (18). The observed decrease in basal heat production was no greater than that expected from the diminished food intake. The basal metabolism did not rise after the alleviation of the neuritic symptoms by administration of yeast concentrate, indicating that the vitamin *per se* had no specific influence upon heat production.

Within recent years interest in the question was renewed by Kinnorsley and Peters (19) who reported an increase of lactic acid in the brains of pigeons showing symptoms of opisthotonos due to vitamin B<sub>1</sub> avitaminosis, this change being especially pronounced in the lower centers. Fisher (20) has extended such studies to other tissues and observed differences in the lactic acid content of heart, liver, and muscle between vitamin B-deficient pigeons and

normal birds after exercise and rest. He suggested that the mechanism of lactic acid removal may be disturbed in vitamin B deficiency.

Another way to approach the problem of carbohydrate utilization as affected by lack of vitamin B is by a study of the glucose tolerance. It is well known that a decrease in tolerance for sugar serves as an early indication of disorder in diseases involving carbohydrate metabolism; *e.g.*, in diabetes. Collazo (21) was the first to publish such tests on dogs, but his animals were suffering from multiple dietary deficiencies, so that great significance can hardly be attached to his results. Eggleton and Gross (22) reported no difference in glucose tolerance between rats deprived of vitamin B and control animals given an adequate amount of this dietary factor. Their results, however, are not convincing, for two reasons: the experiments were controlled only with respect to the vitamin, but not with reference to the accompanying fasting; the blood samples were obtained by sacrificing the animals, so that the value for each point on the blood sugar curve represented the average of the data for different animals killed after the various intervals. The investigation of Lepkovsky, Wood, and Evans (23) is not subject to this criticism. Their studies showed that before the appearance of the *acute* stage of the disease, the glucose tolerance of the rats deprived of vitamin B was practically normal. In the final stages of polyneuritis, however, the blood sugar curves were characterized by a greater rise and a delayed return.

It seemed urgent to investigate this problem in another species (the dog) for two reasons: (1) corroboration of the results of Lepkovsky, Wood, and Evans would indicate that the phenomenon cannot be due to species difference, and (2) the factor of fasting which complicates the picture of vitamin B deficiency can be *very accurately* controlled in the dog. That this is of the utmost importance is demonstrated by the fact that there have been so many reports of a decrease in sugar tolerance associated with fasting *per se*. Illustrative observations that might be cited have been published by Staub (24) on men, Reinhold and Karr (25) on rabbits, and recently by Deuel (26) on dogs. In view of the fact that fasting involves deprivation of *vitamin B* as well as of the other dietary essentials, the question presented itself whether it is

an insufficiency of the vitamin that is primarily responsible for the observed impairment of carbohydrate tolerance.

#### EXPERIMENTAL

Ten dogs were used for this study. The technique of controlling the food and water intakes is described elsewhere (27). The essential feature of the plan was to give the control animals adequate amounts of vitamin B, but to restrict the intake of food and water to the amounts voluntarily consumed by the experimental companions. During the course of these studies it became obvious that the source of the vitamin under investigation must be in the form of a concentrate, the administration of which would not involve the introduction of an appreciable amount of food. Vitamin B was supplied by a concentrate kindly furnished by R. J. Block, or as Yeast Vitamine-Harris Powder.<sup>1</sup>

The glucose was injected intravenously in suitable concentration instead of being administered *per os*, in order to eliminate the variable of intestinal absorption. The investigation of Pierce, Osgood, and Polansky (28) indicates that the absorption of glucose is impaired in animals deprived of vitamin B.

Inasmuch as all the test doses reported in the literature are based on oral administration, it was necessary to determine a dose suitable for injection technique. Because sugar introduced directly into the vascular system is much more rapidly removed, it was planned to follow the blood sugar curve not more than 2 hours, and to give an amount of glucose small enough to allow in a normal dog a return to the preinjection level at the end of an hour. Blood samples were withdrawn from the jugular vein without stasis 15, 30, 60, 90, and 120 minutes after injection of 1 gm. of glucose per kilo of body weight. The tests were made at least 14 hours after a meal.

The blood sugar was determined by the method of Hagedorn and Jensen (29). The protein-free filtrates, however, were prepared according to the Somogyi procedure (30) by treating 1 cc. samples of blood with the precipitants.

Total solids of the blood were also determined as a routine measure. These data will be omitted because the observed

<sup>1</sup> The Harris Laboratories, Tuckahoe, N. Y.



changes in blood sugar could not be accounted for by changes in blood concentration.

### Results

Although sugar tolerance tests were made at least once every week on each animal throughout the experimental period, it will suffice

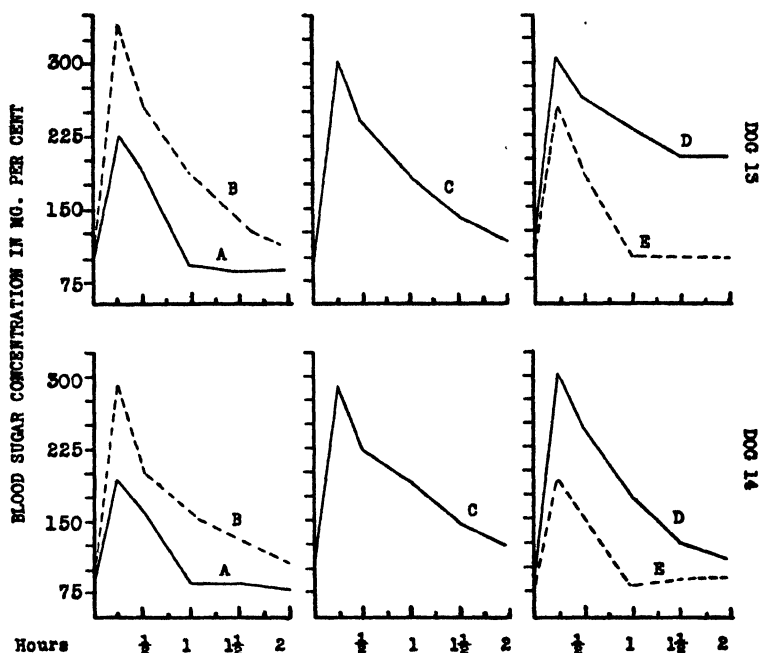


CHART I. The upper diagrams represent five blood sugar curves obtained from a vitamin B-deficient animal (Dog 13) which exhibited a prolonged anorexia. Curve A shows normal response; Curve B, response after 7 days of fasting; Curve C, after 12 days of fasting; Curve D, during polyneuritis; Curve E, after vitamin therapy. The lower diagrams show corresponding blood sugar curves yielded by the control (Dog 14) which received vitamin B but was restricted to the amount of food and water voluntarily consumed by the experimental companion.

here to present a few illustrative curves which show clearly the results of this investigation.

In Chart I are given five sugar tolerance curves taken from an animal deprived of vitamin B (Dog 13) and from the companion control (Dog 14) which received this vitamin but was restricted

to the amount of food and water voluntarily consumed by the experimental companion. Dog 13 represents the type of animal which undergoes severe inanition when vitamin B is withheld. In the upper set of diagrams, Curve A illustrates the normal response of this dog to the injection of the test dose of glucose. 7 days after the onset of anorexia the test made on this animal revealed a definite decrease in the rate at which the injected sugar was removed from the blood (Curve B). The carbohydrate tolerance was not further impaired after 12 days of uninterrupted fasting (Curve C). In the last diagram is shown the very marked decrease in carbohydrate tolerance (Curve D) observed on the day of polyneuritis, the blood sugar level at the end of 2 hours being still very high. After the relief of the symptoms by administration of Vitavose,<sup>2</sup> the response became normal (Curve E).

In the lower set of diagrams (Chart I) are shown the corresponding curves obtained from Dog 14, in which it is obvious that fasting *per se* causes a decrease in carbohydrate tolerance (Curves B, C, and D). However, on the day when symptoms were manifested by the companion Dog 13, the glucose tolerance of the control Dog 14 showed a decrease (Curve D) which was not as marked as that exhibited by the polyneuritic animal. It was of interest to see what effect the ingestion of food would have upon this condition. The animal (Dog 14) was allowed to eat 400 gm. of the ration, and another test was made during the subsequent postabsorptive period (14 hours after the meal). The striking difference in the rate of glucose disappearance is illustrated in Curve E.

That the degree of change in carbohydrate tolerance is related to the nutritive condition of the animal rather than the lack of vitamin B was apparent in those animals which did not exhibit a prolonged anorexia but maintained a fairly good, though erratic appetite. In Chart II, Curve A shows the normal response of Dog 7 to the injection of the test dose of glucose. The character of the blood sugar curve is not significantly changed in Curve B, which was yielded by a test made *only 2 days before symptoms of polyneuritis appeared*. At this time (Curve C) there was evidenced not only a delay in removal of the injected glucose from the blood

<sup>2</sup> A wheat germ preparation made by E. R. Squibb and Sons, New York.

but a rather high initial value. Similarly, Dog 10 exhibited a normal sugar tolerance 4 days before onset of polyneuritis (Curve X). When symptoms were in evidence, however, the blood sugar curve (Curve Y) showed a delay which was much more marked than that exhibited by the companion control. On the day following the administration of Vitavose both dogs (Dogs 7 and 10) gave a normal reaction to the injection of glucose.

When vitamin therapy was instituted in the five polyneuritic animals thus far studied, this dietary essential was supplied by a preparation (Vitavose) which also contained a considerable amount of carbohydrate. It was not known, therefore, whether the

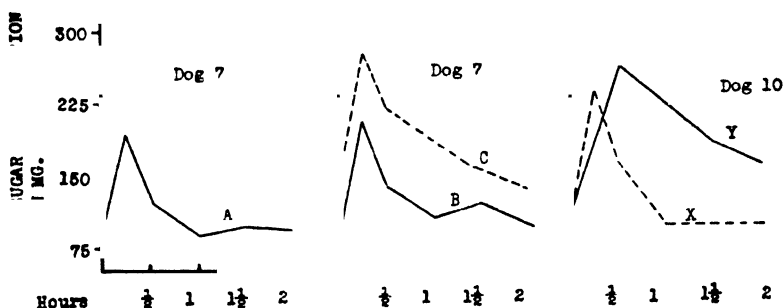


CHART II. Blood sugar curves obtained from dogs subsisting on a vitamin B-deficient diet. Curve A (Dog 7) shows normal response; Curve B, response 2 days before symptoms of polyneuritis; Curve C, during polyneuritis. Curve X (Dog 10) shows response 4 days before onset of disease; Curve Y, during polyneuritis.

improvement in glucose tolerance thus brought about was due to the carbohydrate administered or to the vitamin.

In the latter part of this investigation a concentrate suitable for parenteral injection and containing only 4 per cent of total solids was made available through the courtesy of R. J. Block (31). The vitamin B-deficient animal was made to serve as its own control. A glucose tolerance test was made upon the animal when symptoms of polyneuritis were manifested. The dog was then cured by intravenous injection of this concentrate, but no food was given until another test was performed to see the specific effect of vitamin B upon this function. The animal was then

allowed to eat as much food as it desired, and during a subsequent period a third test was made to determine the effect of food.

Two animals were studied in this way, and the results of both offer definite evidence that lack of vitamin B is not primarily responsible for the observed decrease in sugar tolerance. This

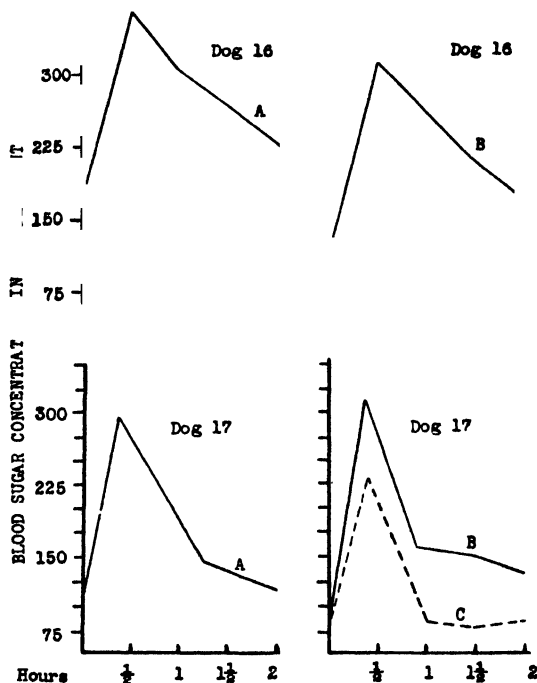


CHART III. Blood sugar curves yielded by two dogs subsisting on a vitamin B-deficient diet (Dogs 16 and 17). Curve A shows response during polyneuritis; Curve B, after the symptoms were removed by intravenous injection of a vitamin B concentrate; Curve C, after the administration of food.

evidence is presented in the blood sugar curves shown in Chart III. Curve A represents the response of Dog 16 to the injection of 1 gm. of glucose per kilo during the polyneuritic stage, in which there is observed a very high initial value of 178 mg. per cent and a delay in the return to the postprandial level. Curve B was obtained from a test made after the relief of the symptoms

by intravenous injection of a vitamin B concentrate. The pre-injection level of blood sugar was found to have decreased to almost normal, but the character of the blood sugar curve was not changed. After the animal received food, however, it exhibited an increase in tolerance which was even greater than that normally displayed (Curve C).

The last animal (Dog 17) was given a larger test dose of glucose (10 gm. for 7.6 kilos of body weight). Unlike the animal discussed above, which exhibited a severe anorexia, this dog maintained an erratic appetite until 4 days before the manifestations of the symptoms, during which period food was completely refused. The decrease in carbohydrate tolerance was not as great as that shown by Dog 16, but the results furnish corroborative evidence that a normal response is not obtained until after the administration of food (compare Curves B and C of Dog 17).

#### DISCUSSION

It was consistently observed that the decrease in tolerance was always greater in the *polyneuritic* animal than in the companion control. However, in view of the fact that those vitamin B-deficient animals which maintained a fairly good appetite for the ration displayed practically normal blood sugar curves until shortly before onset of symptoms, it is suggested that the observed disturbance at this time is part of the polyneuritic syndrome. From a study with rats, Lepkovsky, Wood, and Evans (23) express the same opinion: "it is remarkable that for a considerable time animals may be definitely avitaminotic . . . and yet give glucose tolerance curves nearly identical with those of their controls. . . . The most marked disturbance in glucose tolerance always occurs in the premortal stage."

The effect of fasting upon carbohydrate tolerance has been pointed out by many investigators. The length of time required to bring about this disturbance is too short for possible depletion of vitamin B. For example, Goldblatt (32) observed indications of incomplete carbohydrate oxidation following his fast of as few as 40 hours. Du Vigneaud and Karr (33) demonstrated that progressive reduction in carbohydrate tolerance occurs in rabbits fasted 1 to 4 days. Similar observations were made on dogs by Deuel (26), in which a much greater rise above the initial blood

sugar value and a marked delay in the return to the postprandial level were exhibited in dogs which had been fasted 3 to 4 days, as compared with a normal response. It is inconceivable that lack of vitamin B should be the responsible factor here; it has been the experience of this laboratory that a normal dog has a readily available store of this vitamin which is not depleted until after about 3 weeks upon the vitamin B-deficient ration. Furthermore, Deuel demonstrated that the response to glucose administration becomes normal when the test is preceded about 14 hours by the ingestion of a large amount of carbohydrate (150 gm. of sucrose).

What is the nature of the mechanism which is so quickly impaired by carbohydrate starvation? Cori and Cori (34) observed that insulin completely restored the oxidation of sugar in rats fasted for 48 hours, and concluded that the insulin production mechanism is related to this disturbance. A similar view is held by Tolstoi (35), who studied the glucose tolerance of two men fed diets poor in carbohydrate. The current theory, therefore, which is offered to account for this "hunger diabetes" is that there is a gradual decrease in insulin production by the pancreas during carbohydrate starvation, which function cannot be adjusted quickly enough to meet the sudden demand that arises when an excess of carbohydrate is introduced.

Dann and Chambers (36) reported experiments designed to investigate this theory. They fasted dogs for a period of 19 to 31 days and determined the respiratory quotient of the fasting animals. The dogs were then allowed to ingest a given amount of glucose and observations of the metabolism were made during a subsequent period. They found that when insulin was injected concomitantly with the ingestion of glucose, "only a partial compensation for the loss of ability to metabolize carbohydrate" was produced. They concluded that some other factor (or factors) was involved in the phenomenon. It is pertinent to point out that dogs which have been fasted as long as those of Dann and Chambers are usually depleted of vitamin B sufficiently to bring about the anorexia so characteristic of lack of this substance. However, the results of the investigation here reported do *not* indicate that vitamin B is the factor supplementing insulin in the regulation of carbohydrate metabolism.

## SUMMARY AND CONCLUSIONS

A study was made of the glucose tolerance of dogs subsisting on a diet lacking undifferentiated vitamin B. The effects of the concomitant fasting were adequately controlled.

A definite decrease in glucose tolerance was exhibited by both the vitamin B-deficient animals and the controls. The degree of the disturbance was directly proportional to the severity of the fasting.

In five polyneuritic animals the vitamin was administered in the form of a preparation containing a considerable amount of carbohydrate, so that it was not possible to conclude whether the observed improvement in glucose tolerance was due specifically to the vitamin.

In two experiments a concentrate of the antineuritic factor practically devoid of carbohydrate and suitable for parenteral administration was available. Intravenous injection of this product was made into the dogs exhibiting the characteristic nervous manifestations of vitamin B deficiency. Despite the removal of the symptoms by this concentrate there was no significant effect upon the carbohydrate tolerance. Ingestion of food, however, was promptly followed by a return of the glucose tolerance to normal.

It is concluded that the decrease in carbohydrate tolerance shown by animals deprived of vitamin B is the result of the accompanying inanition. The view that vitamin B plays a specific rôle in the metabolism of carbohydrate is not supported by this investigation.

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## THE METABOLISM OF TRYPTOPHANE

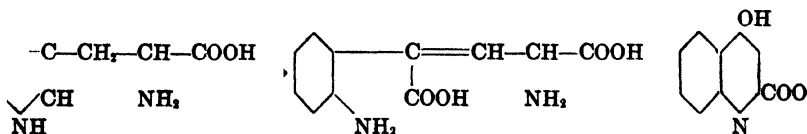
### III. THE AVAILABILITY OF KYNURENINE IN SUPPLEMENTING A DIET DEFICIENT IN TRYPTOPHANE\*

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The current information concerning the excretion of kynurenic acid by certain species of animals apparently justifies the prevailing conception that this substance is not a link in the chain of normal tryptophane oxidation in the animal body, but rather that it is an end-product of a set of side reactions brought into play especially when tryptophane is administered in excess of ordinary metabolic requirements. Several investigators (Ellinger and Matsuoka, 1914, 1920; Barger and Ewins, 1917; and Robson, 1924-25, 1928) employed singularly ingenious methods in attempts to elucidate the nature of these reactions. It remained, however, for Matsuoka and Yoshimatsu (1925) and Kotake and his associates (1931) to secure the first conclusive evidence as to the mechanism in question. The introduction of large amounts of tryptophane into rabbits was found to lead to the excretion of not only kynurenic acid but also a hitherto unknown metabolic product. The latter was isolated, purified, and apparently correctly identified. The substance, an amino acid termed *Kynurenin*, is convertible to kynurenic acid *in vitro* as well as *in vivo*. Without reciting the many interesting details of the report of the Japanese investigators, one may summarize their findings by inserting kynurenine as a definite step in the tryptophane-kynurenic acid transformation as follows:



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† National Research Council Fellow in Medicine, 1930-31.

The investigation described herein was undertaken to determine whether or not the reactions whereby kynurenine is synthesized from tryptophane are reversible to any appreciable degree in the animal organism. The fact that 20 to 40 per cent of the kynurenine administered to rabbits may be recovered as kynurenic acid does not preclude the possibility of kynurenine playing a vicarious rôle in the diet of animals placed under stress for tryptophane. Inasmuch as tryptophane under suitable conditions gives rise to kynurenine, a still more intimate physiological connection between the two substances would be established if kynurenine could be shown to serve in lieu of tryptophane in the nutrition of growth. This relation in turn would suggest that the tryptophane degradation resulting in kynurenic acid and the catabolism terminating in less specific end-products *might* travel a common chemical pathway as far as the kynurenine stage.<sup>1</sup>

Asayama (1916) found that kynurenic acid did not restore growth to albino rats deprived of tryptophane. This observation is perhaps not surprising in view of the fact that kynurenic acid, a derivative of a stable ring system different from that in tryptophane, is produced from the latter amino acid by a series of alterations including complete loss of 1 carbon atom. Reversal of the process is difficult to conceive. Kynurenine, on the other hand, differs from tryptophane only by an incipient oxidation and a hydrolysis of a resulting lactam ring. Resynthesis would require in this instance simply a closing of the ring by loss of water and subsequent appropriate reduction.

#### EXPERIMENTAL

Kynurenine was prepared and fed as the sulfate. The urines of rabbits were collected following the administration of large doses of tryptophane. From these urines acidified with sulfuric acid, the kynurenine sulfate was precipitated by means of alcohol (Kotake *et al.*, 1931). 2 gm. of a very slightly colored but nicely

<sup>1</sup> It is noteworthy that the rôle of kynurenine as a physiological intermediary in the formation of urinary pigment has been investigated by Kotake and his associates. Referring to their studies on the origin of urochrome, these authors state: "Die vorliegenden Resultate sprechen alle dafür, dass das Urochromogen oder Urochrom aus dem Tryptophan und zwar über das Kynurenin gebildet wird."

crystalline preparation were treated with bone-black and recrystallized from 50 to 75 cc. of hot water. The product amounting to 700 mg. consisted of pure white needles which darkened at about 175° and sintered in the neighborhood of 185°. The crystals did not definitely melt even at 200°. An analysis is presented in the accompanying tabular matter for the purpose of establishing identity with the substance  $C_{11}H_{12}O_4N_2 \cdot H_2SO_4$  described in the literature cited above.

	Calculated	Author found	Kotake <i>et al.</i> found
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Carbon.....	39.50	38.86	
Hydrogen.....	4.23	4.80	
Nitrogen.....	8.38	8.54	8.51
Sulfuric acid as sulfur.....	9.60	10.13	9.82

Inasmuch as the procedure used for testing the effect of kynurenine sulfate on the growth of albino rats was substantially the same as that used in a similar investigation by the writer (1929), a description of most of the experimental details is omitted here. The animals, all males from one litter, at a weight of about 100 gm. each were transferred from an adequate food to that deficient in tryptophane. This diet consisted of acid-digested casein 14.4, tyrosine 0.3, cystine 0.3, dextrin 39, sucrose 15, salts (Osborne and Mendel, 1919) 4, ground agar 2, and lard 25 per cent. 250 mg. of dried yeast and 100 mg. of cod liver oil were fed daily apart from the remainder of the diet. 250 mg. of tryptophane were chosen as 1 equivalent for inclusion in 100 gm. of basal food. The 50 mg. of this amino acid actually used in the control experiments are, therefore, designated as 0.2 equivalent. The kynurenine sulfate was fed comparably on the basis of molecular weight in 1 and 2 equivalent amounts; *viz.*, 410 and 820 mg. per 100 gm. of basal diet.

#### DISCUSSION AND SUMMARY

It is apparent from Chart I that there is no effect on growth when kynurenine sulfate is fed over a period of 2 weeks in amount 5 and 10 times that equivalent to a quantity of tryptophane which produces an immediate sharp upward inflection in the body weight

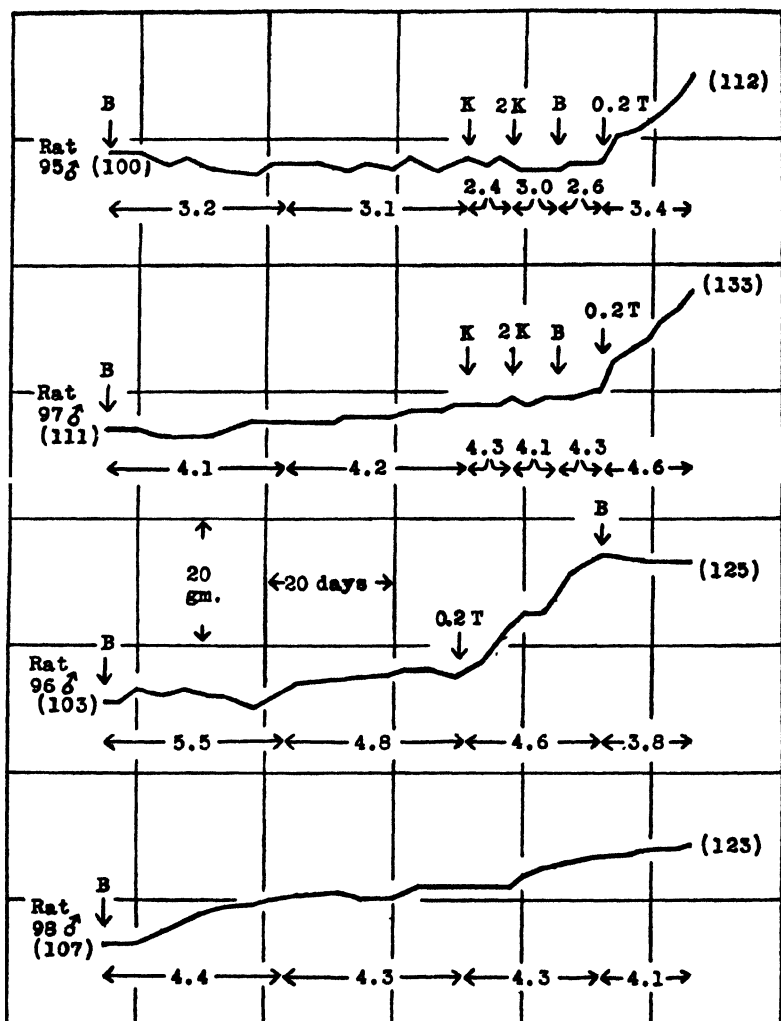


CHART I. Growth on kynurenine sulfate. Rats 95 and 97: growth on basal tryptophane-deficient Diet B (B); on Diet B with 1 equivalent (K) and 2 equivalents (2K) of kynurenine sulfate; on Diet B; and finally on Diet B with 0.2 equivalent of tryptophane (0.2T). Rats 96 and 98 are controls. Any abscissa value represents the same day for all four curves. The average daily food consumption in gm. for various periods is indicated just below each corresponding curve. Initial and final body weights are shown in parentheses.

graph. It should be noted that the kynurenine prepared as described is optically active and therefore might possibly be the unnatural enantiomorph. However, inasmuch as the asymmetric carbon atom is apparently unaltered during the animal synthesis, we may presume that the configuration of that carbon atom in kynurenine is the same as that of *l*-tryptophane. At any rate, Berg and Potgieter (1932) have shown that *dl*-tryptophane serves about as well for growth function as does *l*-tryptophane. Finally, it must be observed that the experiments reported here like others of their kind are subject to such undetermined factors as failure of absorption, bacterial decomposition, hepatic alteration, etc. In other words the introduction of a substance into the gut is not equivalent to its transfer into the cells at large in the body. With these limitations in mind, one may draw the conclusion that kynurenine is not transformed into tryptophane by reverse synthesis in the animal organism.

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# THEORY OF THE REVERSIBLE TWO-STEP OXIDATION

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## *I. Fundamental Theory*

It has been shown in previous papers (1-3) that a reversible oxidation-reduction process in certain dyestuffs can take place in two successive steps, each characterized by a particular level of the oxidation-reduction potential. Each step involves the detachment or acceptance of 1 electron.<sup>1</sup> The intermediary compound has the constitution of a chemical radical, a molecule with an odd number of electrons. The results obtained with pyocyanine by Friedheim and Michaelis (1) were very soon confirmed by Elema (4) who independently of these authors worked on the same subject. Following the presentation in the quoted papers of the experimental facts and conclusions drawn from them, as far as it was possible without an elaboration of the theory, it will be the task of this paper to develop more thoroughly the theory of the reversible two-step oxidation-reduction process and especially of the potential curve obtained on titrating the reduced form of the dye with an oxidant, or the oxidized form with a reductant, as the case may be. We restrict ourselves to the case that the intermediary form is what we defined as a semiquinone. The case of a meriquinone has been discussed by Clark, Cohen, and Gibbs (5). The case of a semiquinone has been briefly discussed by Elema whose results and formulæ, however, are not identical with those to be presented in this paper. The theory to be presented

<sup>1</sup> In the previous paper (2) the case of two-step oxidation described by Cannan for hermidine was quoted as another example of this kind. This was a mistake of mine which I wish to correct on this occasion. In the case of hermidine, according to Cannan, each step of the oxidation involves 2 electrons.

will be applicable even when the two steps of oxidation overlap, whereas the simplified theory such as is used in the preceding papers (1, 2) holds only when the two steps do not noticeably overlap.

Because of the complicated nature of the theory it seemed advisable to devote this paper only to the development of the theory and thus to give all theoretical preparations, the application of which will be treated in subsequent papers concerned with the experimental data.

Let us designate the three forms of the substance as  $R$  (the reduced form),  $S$  (the semiquinoid or intermediary form), and  $T$  (the totally oxidized, or quinoid, or holoquinoid form). The amount, in mols, of these three substances will be designated respectively by  $r$ ,  $s$ ,  $t$ .

Let the substance in its completely reduced form be titrated by a strong oxidant. Let the initial amount of  $R$ , in mols, be  $a$ . During the titration  $R$  will gradually disappear and  $S$  and  $T$  will be formed. During the whole titration the following relation will hold.

$$r + s + t = a \quad (1)$$

We assume that all oxidations and reductions consist only in the loss or acceptance of electrons, not hydrogen atoms. In a later chapter of this paper this simplifying assumption will be dropped, but first we develop the theory for this most simple condition. The added amount of the oxidant will be designated as  $x$ , the units being chosen in such a way that each step of the titration consumes  $a$  units of the oxidant. Thus,  $\frac{x}{a} \times 100$  indicates the percentage of oxidation for one step. The whole oxidation, then, is completed with 200 per cent, when  $x = 2a$ . At the end of the first step  $x = a$ . At any point of the titration, there will be

$$s + 2t = x \quad (2)$$

A third equation is obtained by taking into account the fact that there must be an equilibrium established for the following reversible chemical reaction.





Such a reaction is often called a dismutation. Its equilibrium is determined by

$$s^2 = k \cdot r \cdot t \quad (3)$$

It is easy to see why in this equation the absolute amounts,  $r$ ,  $s$ ,  $t$ , can be used instead of the concentrations  $[r]$ ,  $[s]$ ,  $[t]$ . (This could not be done if we had to deal with a meriquinone instead of a semiquinone.)  $k$  is the equilibrium constant to which we will refer as the *formation constant* of the semiquinone. Its reciprocal value may be called the *dismutation constant*. These three equations, Equations 1, 2, and 3, can be solved for  $r$ ,  $s$ , and  $t$  in terms of  $a$ ,  $x$ , and  $k$ . The result is

$$r = a \left( 1 + \frac{p}{2} \right) - \frac{x}{2} \mp \frac{1}{2} \sqrt{Y} \quad (4)$$

$$s = -a \cdot p \pm \sqrt{Y} \quad (5)$$

$$t = a \cdot \frac{p}{2} + \frac{x}{2} \mp \frac{1}{2} \sqrt{Y} \quad (6)$$

where

$$4 - k \quad (7)$$

and

$$Y = a^2 p^2 + 2apx - px^2 \quad (8)$$

Only one of the two signs before the square root has a physical significance, but it cannot be decided once for all which one holds. A closer study of this function reveals that the upper sign must be chosen when  $k < 4$ , and the lower sign when  $k > 4$ , in order to obtain results of a sensible physical meaning. This fact has an intrinsic connection with the character of the parameter,  $p$ , as defined in Equation 7. When  $p$  is plotted against  $k$ , the point  $k = 4$  turns out to be a point of discontinuity,  $p$  jumping from  $+\infty$  to  $-\infty$ .

If  $k$  is known,  $r$ ,  $s$ , and  $t$  can be calculated for each value of  $x$ , and the potential is then determined by any one of these three equations:

$$E = E_m + \frac{RT}{2F} \ln \frac{t}{r} \quad (9)$$

or

$$E = E_1 + \frac{RT}{F} \ln \frac{s}{r} \quad (10)$$

or

$$E = E_2 + \frac{RT}{F} \ln \frac{t}{s} \quad (11)$$

When both sides of Equation 4, 5, or 6, are divided by  $a$ , the right-hand sides contain the magnitudes  $x$  and  $a$  only in form of the ratio  $\frac{x}{a}$ . This shows that  $r$  (or  $s$  or  $t$ ) when measured as a fraction of  $a$  does not depend on the volume of the solution. The same holds for the potentials. This is in contrast to what would be the case if the intermediary form were a meriquinone instead of a semiquinone.

$E_m$  is the potential in the mid-point of the titration when  $x = a$ . The meaning of  $E_1$  and  $E_2$  is, in general, not quite so simple. But for all those cases where the two steps of the titration are very distinctly separated, in other words, where  $E_2$  is very much more positive than  $E_1$ ,  $E_1$  is the potential in the mid-point of the first half, and  $E_2$  the one in the mid-point of the second half of the titration.

It is appropriate to term  $E_1$  as the normal potential of the first step,  $E_2$  as the normal potential of the second step, and  $E_m$  as the normal potential of the whole system. It is easy to infer from Equations 9, 10, and 11 that  $E_m$  is the mean value of  $E_1$  and  $E_2$ , or

$$E_m = \frac{E_1 + E_2}{2} \quad (12)$$

Not only the sum, but also the difference of the normal potentials of the two steps has a particular significance. For the difference  $E_2 - E_1$  has a definite relationship to  $k$ . According to Equations 10 and 11

$$E_2 - E_1 = \frac{RT}{F} \ln \frac{s^2}{r \cdot t}$$

and, using Equation 3, we find

$$E_2 - E_1 = \frac{RT}{F} \ln k$$

or, for 30°

$$\log k = \frac{E_2 - E_1}{0.0601} \quad (13)$$

So, *e.g.*, when  $E_1 = E_2$ ,  $\log k$  will be equal to 0, or  $k = 1$ . This is a striking result. One might be sure that anybody not thoroughly familiar with the subject would have guessed that  $k = 0$  (instead of 1) when  $E_2 = E_1$ .

According to the above equations one can calculate the potential as a function of  $x$ , when  $k$  is known. Or, conversely, from the

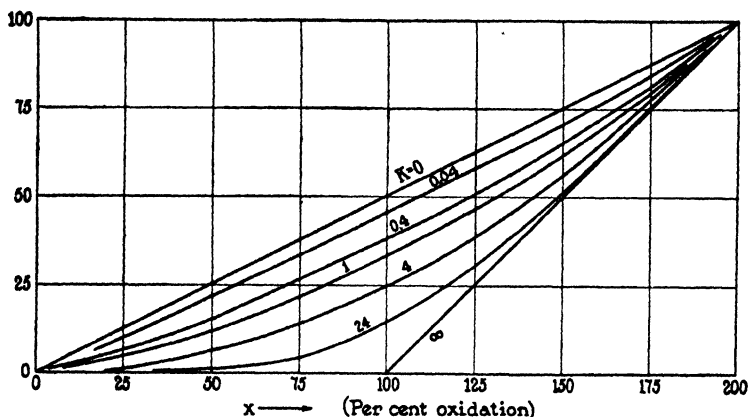


FIG. 1.  $t$ , the totally oxidized form (or quinoid, or holoquinoid form) in per cents of the total amount of the dye plotted against per cent of oxidation. When the scale of the abscissa is reversed, it is  $r$ , the reduced form plotted against per cent of oxidation.

potentials observed, one can calculate  $k$ . To evaluate such a calculation it is instructive to plot  $r$ ,  $s$ ,  $t$ , and  $E$  against  $x$  for varied values of  $k$ , because the knowledge of these magnitudes is requisite for the calculation of the potentials. In all these calculations,  $a$  will be set equal to 100, so that  $x$  is the percentage of oxidation for one step. At the end-point of the whole titration  $x$  will then be equal to 200. The result of the computations is shown in the graphs, Figs. 1 to 3. For  $k = 4$ ,  $p$  becomes  $\pm \infty$ . In this case the functions cannot be directly evaluated, but they can be interpolated between those for values somewhat greater and somewhat smaller than 4.

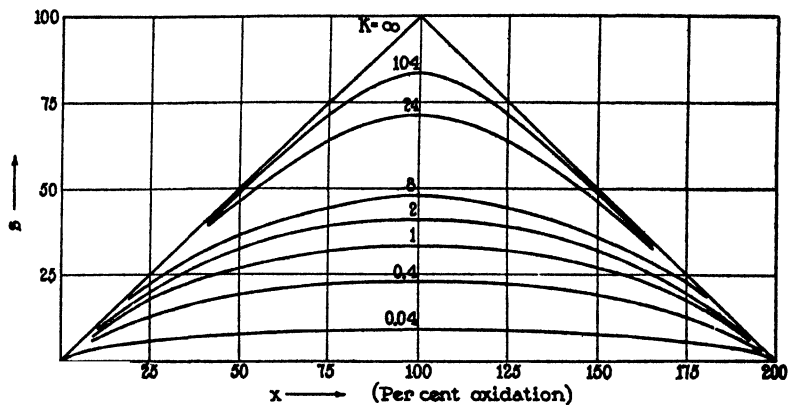


FIG. 2.  $s$ , the semiquinoid or intermediary form in per cents of the total amount of the dye plotted against per cent of oxidation.

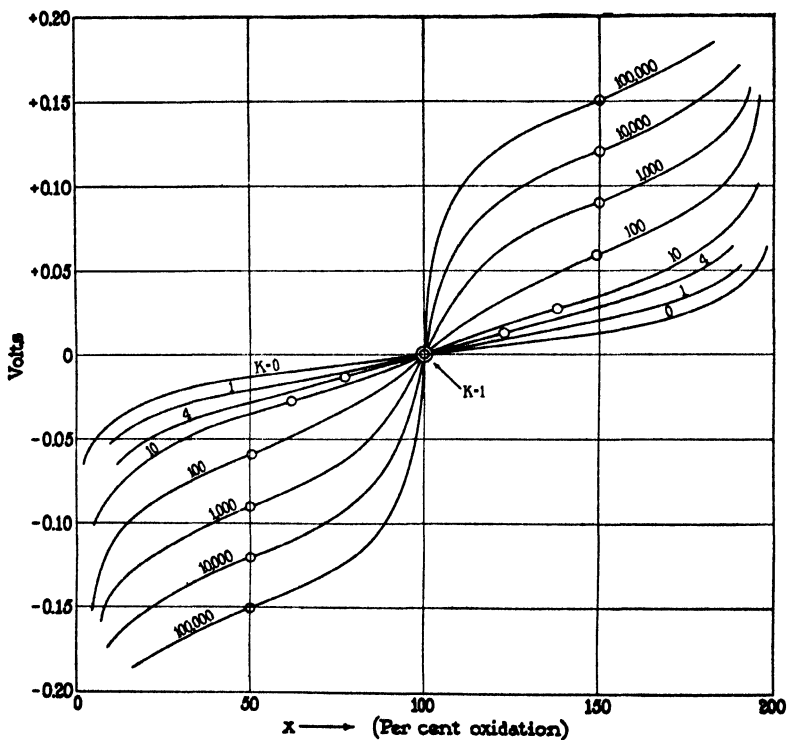


FIG. 3. The potential (in volts) plotted against per cent of oxidation

Fig. 1 shows the oxidized form,  $t$ , in per cents of the sum of all three forms, plotted against per cent of oxidation. Each curve of the family holds for the value of  $k$  indicated. The same figure can be taken as a graph for  $r$  by reversing the scale of the abscissa (200 in place of 0, and 0 in place of 200).

Fig. 2 shows the semiquinone,  $s$ , in per cents of the sum of all three forms, plotted against per cent of oxidation, for varied values of  $k$ .

Fig. 3 shows the potential plotted against per cent of oxidation. The potential in the mid-point of the titration is taken as 0 for all curves. Each curve holds for the indicated value of  $k$ . The circles indicate that point of the curve at which the potential is equal to  $E_1$  (on the left-hand side of the graph) or  $E_2$  (on the right-hand side). The double circle in the mid-point of the graph is the point where in the curve  $k = 1$  the values of  $E_1$  and  $E_2$  coincide. No curves have been drawn between  $k = 1$  and  $k = 0$ . For such curves,  $E_2$  is more negative than  $E_1$ , in contrast to all curves for  $k > 1$ . In these curves,  $E_2$  would lie on the left-hand side, and  $E_1$  on the right-hand side.

#### *Particular Solution of Problem for the Case $k = 4$*

The general solution of the problem as presented in Equations 4 to 11 cannot be directly applied for the particular case when  $k = 4$ , as stated above. We may apply the method of interpolation for this special case, but it is more satisfactory and quite easy to obtain a particular solution of the problem for the case  $k = 4$ . When we put  $k = 4$  in Equation 3 and solve Equations 1, 2, and 3, we obtain directly the following simple solutions.

$$r = \frac{(2a - x)^2}{4a}; s = \frac{x(2a - x)}{2a}; t = \frac{x^2}{4a}$$

The potential, then, is

$$\begin{aligned} E &= E_m + \frac{RT}{2F} \ln \frac{t}{r} \\ &= E_m + \frac{RT}{2F} \ln \frac{x^2}{(2a - x)^2} = E_m + \frac{RT}{F} \ln \frac{x}{2a - x} \end{aligned}$$

This is a remarkable result. It shows that when  $k = 4$ , the

titration curve has the same shape as a one-step titration curve with the electron number 1 (*e.g.* ferricyanide-ferrocyanide).

In such a case the two-step nature of the system cannot be recognized by the shape of the titration curve alone. Yet it can be distinguished from an ordinary one-step system by the fact that in a two-step system an intermediary color arises during the titration, which is neither the color of the reduced form nor that of the oxidized. On the other hand, when a dyestuff which is known to accept 2 hydrogen atoms on reduction presents a titration curve resembling the curve for a system in which the oxidized and the reduced forms differ only by 1 hydrogen atom, then we have to deal with a two-step oxidation, the formation constant of the semiquinone being equal to 4.

## II. Amplification of Theory

Now we will drop the simplifying condition that the oxidized and the reduced forms differ only by electrons. We must take into consideration that protons (hydrogen ions) can be attached or detached besides. In other words we will take into account the acidic and basic properties of the various forms of molecules. Then Equations 1 and 2 will remain valid provided we understand by  $r$  the sum of all molecules of the reduced form in all possible states of acidic or basic dissociation, and, correspondingly, by  $s$  all forms of the semiquinone, and by  $t$  all forms of the totally oxidized form. But Equation 3 holds only for such of the three forms as differ from each other only by electrons. When we pick out among all possible forms of  $r$  one particular form  $r'$ , and among all forms of  $s$  one particular form,  $s'$ , and among all forms of  $t$ , one particular form,  $t'$ , in such a way, that

$$t' + e = s'; s' + e = r'$$

then, instead of Equation 3, we obtain  $s'^2 = k \cdot t' \cdot r'$ . According to the mass action law,  $t'$  is a function of  $t$ , depending on the hydrogen ion concentration,  $h$ , and all the dissociation constants concerned. Without discussing the nature of these functions in detail we may express this relationship generally in a symbolic manner as follows:

$$\begin{aligned} t' &= t \cdot f_1 (h, k_{11}, k_{12} \dots) \text{ and analogously} \\ s' &= s \cdot f_2 (h, k_{21}, k_{22} \dots) \text{ and} \\ r' &= r \cdot f_3 (h, k_{r1}, k_{r2} \dots) \text{ or briefly} \\ t' &= t \cdot f_1; s' = s \cdot f_2; r' = r \cdot f_3 \end{aligned}$$

and Equation 3 now reads

$$s^2 = \frac{k \cdot f_1 \cdot f_3}{r \cdot t}$$

The magnitude  $\frac{s^2}{r \cdot t}$  which was a constant for the simplified condition in Equation 3 now becomes a variable.

$$\frac{f_1 \cdot f_3}{f_2^2} = K \quad (3, a)$$

When this  $K$  is used instead of  $k$ , all the equations from Equation 4 to 11 remain valid. The amplification of the theory, therefore, consists only in the fact that a magnitude  $K$  which depends on pH must be used instead of the constant  $k$ . We may designate  $K$  as the effective formation constant of the semiquinone, in contrast to  $k$ , the true one. Equation 13 has now to be changed into

$$\log K = \frac{E_2 - E_1}{0.0601} \quad (13, a)$$

and so the difference of the two normal potentials of the two steps also becomes dependent on pH. This statement implies that the curve for  $E_1$  and that for  $E_2$ , plotted against pH, need not be parallel and may even intersect. The curve  $E_m$  will always stand midway between  $E_1$  and  $E_2$  according to the necessary postulate

$$E_m = \frac{E_1 + E_2}{2} \text{ but otherwise the paths of these three curves may}$$

follow any course. This course will be determined by the dissociation constants in the way outlined by Clark and Cohen (6). Each dissociation constant will bring about a change in the slope; either upward (when this constant belongs to the reduced form of that particular oxidation-reduction system), or downward (when this constant belongs to the oxidized form). The change in the slope will amount to 0.06 volt per unit of pH when the oxidized and reduced forms differ by 1 electron. The change will

be 0.03 volt per unit of pH when the oxidized and the reduced forms differ by 2 electrons. Obviously  $E_m$  is a 2-electron curve,  $E_1$  and  $E_2$  are each 1-electron curves. To give an example, a dissociation constant of the totally oxidized form will manifest itself by bends in the  $E_2$  and  $E_m$  curves but not in the  $E_1$  curve. The  $E_2$  curve will, owing to this dissociation constant, suffer a change of slope by 0.06 volt per unit of pH, and the  $E_m$  curve will suffer a change of slope by 0.03 volt, and the  $E_1$  curve will suffer no change at all at the pH corresponding to that particular dissociation exponent of the totally oxidized form. It is now easy to imagine the various kinds of possibilities but it will not be necessary to state all details which may occur. It is preferable to leave the general considerations at this point and to treat each case which may be encountered in practice in an individual manner as required by the experimental data.

### *III. Adaptation of Theory to Practical Use. The Index Potential*

In the practical application of the formula for the potential, Equations 9 to 11, great difficulty is encountered, owing to the very involved algebra. When the two steps of oxidation are sufficiently separated the simplified calculation as used in the previous papers (1, 2) is satisfactory. Only when the overlapping of the two steps is obvious, does the complicated theory, such as developed in this paper, become requisite. The difficulties in algebra can be circumvented in a certain way and the practical solution of the problem will be as follows:

From Fig. 3 it can be seen, that within the family of these curves with a common mid-point (designated by the double circle) nowhere does an intersection of any two adjacent curves take place, except at this common mid-point. Hence it follows that when the mid-point and any one other point of a curve are given, the whole curve is unequivocally determined. From this principle we arrive at the following argument.

Let us introduce three auxiliary magnitudes,  $E_{\frac{1}{2}}$ ,  $E_{\frac{3}{4}}$ , and  $E_{\frac{1}{4}}$ . We mean by  $E_{\frac{1}{2}}$  the potential in the mid-point of titration, when half the oxidant necessary for complete oxidation has been used up, and by  $E_{\frac{1}{4}}$  the potential at one-quarter of the total oxidation, and by  $E_{\frac{3}{4}}$  the potential at three-quarters of the total oxidation. No further proof is needed that in any case  $E_{\frac{1}{2}} = E_m$ . Further-



more, when the separation of the two steps is very great,  $E_1 = E_1$ , and  $E_1 = E_2$ . But these two equations do not hold when the two steps overlap. When we deal with an ordinary one-step titration with the electron number 2, as in most organic dyestuffs, there will be, for 30°

$$E_2 - E_1 = \frac{0.0601}{2} \cdot \log 3 = 0.0143 \text{ volt}$$

For, in the mid-point of the titration the ratio of the oxidized and the reduced forms is 1:1, and at one-quarter of the oxidation this ratio is 1:3; hence,

$$E_2 - E_1 = 0.03 \log \frac{1}{1} - 0.03 \log \frac{1}{3} = 0.03 \log 3 = 0.0143 \text{ volt}$$

In the same way also

$$E_2 - E_1 = 0.0143 \text{ volt}$$

Whenever a step formation takes place, this magnitude will be  $> 0.0143$  volt. Any step formation will manifest itself by the fact that on considering the titration curve as graphically interpolated from a titration experiment

$$E_2 - E_1 > 0.0143 \text{ volt}$$

We will refer to this magnitude as the index potential,  $E_i$ , of the titration curve because it indicates the step formation. The definition is, therefore,

$$E_i = E_2 - E_1 = E_2 - E_1 \quad (14)$$

Using Equations 9 to 11, and setting  $x = a$ , or  $x = \frac{a}{2}$ , one can, once for all, calculate  $E_1$  and  $E_2$ , hence also  $E_i$  for various values of  $K$ . Table I shows the result of this calculation. On account of the difficult algebra the calculation has been performed only for such values of  $K$  as make the magnitude  $p$  of Equation 7 a simple integer or otherwise convenient figure. These exactly calculated values were utilized in a graphic interpolation and the result of this interpolation is shown in Table I. One may be sure that the

uncertainty of these interpolated values will never exceed 0.1, or, at the worst, 0.2 millivolt, and therefore that the calculations will be sufficiently accurate for practical use. As  $K$  may be determined by means of Table I from  $E_i$ , the difference of the two normal potentials,  $E_2 - E_1$ , can be calculated according to Equation

TABLE I

log $K$	0.03005 log $K$	$E_i$
$-\infty$		0.0143
-2	-0.0601	0.0147
-1.5	-0.0451	0.0155
-1	-0.0300	0.0168
-0.8	-0.0240	0.0174
-0.6	-0.0180	0.0181
-0.4	-0.0120	0.0191
-0.2	-0.0060	0.0203
$\pm 0$	$\pm 0$	0.0218
+0.2	+0.0060	0.0237
0.4	0.0120	0.0258
0.6	0.0180	0.0286
0.8	0.0240	0.0320
1.0	0.0300	0.0362
1.2	0.0361	0.0404
1.4	0.0421	0.0448
1.6	0.0481	0.0494
1.8	0.0541	0.0546
2.0	0.0601	0.0601

The first column is the logarithm of  $K$ , the "effective formation constant of the semiquinone," according to the definition in Equation 3,  $\alpha$ . The second column gives the product of this log  $K$  by the factor 0.03005. The third column,  $E_i$ , is the "index potential," or the difference of the potential at the mid-point of the whole titration and at the mid-point of the first half of the titration, according to the definition in Equation 14. Whenever log  $K > 2$ , then  $E_i = 0.03005 \log K$ .

13.a. The half of this magnitude,  $\frac{E_2 - E_1}{2}$ , can be read from the second column of Table I.

Table I is what we may call the extract, for practical use, from the whole theory. The table can be used, once for all, in translating the index potential, such as is directly measured in the graph

of the titration experiment, into the difference of the two normal potentials of the two steps of oxidation, and into the effective formation constant of the semiquinone,  $K$ . We rely here on the location of one single point of the curve and its relation to the mid-point of the curve. This is permitted only when evidence has been given in some way or other that the titration curve is really a member of this family. This postulate is equivalent to saying: It must be shown that the intermediary step is a semiquinone according to the definition previously given (1, 2) and not a meriquinone or anything else.

On comparing the column  $0.03 \log K$  and the column  $E_i$ , one can see that with increasing values of  $\log K$  the difference of these two values becomes smaller and, from  $\log k = 2$  on, can practically be neglected. From here,  $E_1$  coincides with  $E_i$ , or the separation of the two steps of oxidation is practically complete.

#### SUMMARY

The theory of the two-step oxidation in organic dyestuffs is developed for the case of semiquinone formation. Three different oxidation levels,  $R$  (reduced form),  $S$  (the semiquinoid or half oxidized form), and  $T$  (the totally oxidized, or quinoid, or holoquinoid form), are distinguished and used as the basis for the calculation of the potential. A distinction is made between the true ( $k$ ) and the effective ( $K$ ) formation constant of the semiquinoid form and it is shown that  $k$  is independent of, but  $K$  dependent on, the pH. This is the reason why the formation of the intermediary form and the separation of the two levels varies with pH. As the practical application of the formulæ arising from this theory is, in general, cumbersome, owing to the involved algebra, a substitute is shown for practical use, which takes advantage of a magnitude designated as the index potential.

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# **GALACTOSE TOLERANCE OF NORMAL AND DIABETIC SUBJECTS, AND THE EFFECT OF INSULIN UPON GALACTOSE METABOLISM.**

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It is the purpose of this paper to present the results of an investigation of galactose metabolism by determining the galactose content of the blood and urine following the administration of galactose by mouth to human subjects, and by determining the galactose concentration of the blood following the subcutaneous, intravenous, or intraperitoneal administration of galactose to animals. Investigations of galactose metabolism by similar procedures have been carried out by Corley (1), Blanco (2), Harding and van Nostrand (3), and Harding and Grant (4).

The analytical method used to determine the blood galactose is an application of the procedure of Somogyi (5), in which the fermentable sugar of the blood is removed by treatment with yeast, and of the new Benedict (6) method for determining blood glucose in tungstomolybdic acid filtrates. The urinary galactose was determined by treating the urine with yeast for  $\frac{1}{2}$  hour or more, centrifuging the mixture, and titrating the supernatant fluid against Benedict's quantitative copper reagent.

## *Method for Determination of Galactose in Blood*

Pipette 1 cc. of blood into a small Erlenmeyer flask. Add 7 cc. of 10 per cent washed yeast suspension. Shake the flask to mix the contents thoroughly and place it in an incubator at 37–40° for 15 minutes. The flask should be shaken two or three times during the incubation period to bring about a resuspension of the yeast in the blood. After 15 minutes, remove the flask from the incubator and add 1 cc. of Benedict's tungstomolybdate solu-

tion (7). Add 1 cc. of 0.62 N sulfuric acid, mix thoroughly, and filter through a filter paper which has previously been washed free of soluble reducing substances. Pipette 2 cc. of the filtrate into a Folin-Wu blood sugar tube. Two galactose standards are now prepared. In one Folin-Wu tube place 1 cc. of standard galactose solution containing 0.1 mg. of galactose per cc. and add 1 cc. of distilled water. In another Folin-Wu tube place 2 cc. of the standard galactose solution. To the two standard tubes and the tube containing blood filtrate add 2 cc. of the Benedict copper reagent (6). Place the tubes in a boiling water bath for 6 minutes. Remove the tubes, cool, and add to each tube 2 cc. of the Benedict phosphomolybdic acid color reagent (6). Shake the tubes vigorously until the contents are mixed, and dilute to 12.5 cc. Compare the colored solutions in a colorimeter, with the galactose standard which most closely matches the unknown.

*Calculation*—The number of mg. of galactose per 100 cc. of blood is equal to 1000 divided by the reading of the unknown if the 0.1 mg. galactose standard was used, or 2000 divided by the reading of the unknown if the 0.2 mg. standard was used.

*Reagents and Discussion of Method*—The preparation of the tungstomolybdate solution, the alkaline copper sulfate reagent, and the phosphomolybdic acid reagent is described in the papers by Benedict (6, 7). The yeast (Fleischmann's) is washed by suspending in distilled water in centrifuge tubes, centrifuging, and decanting the supernatant fluid as described by Somogyi (5); we found three washings sufficient to remove the soluble reducing substances. Benedict (8) has called attention to the presence of soluble reducing substances in different grades of filter paper. We remove the soluble reducing substances from the filter paper used in this method by washing with water. This is conveniently done by placing the filters in a large beaker and covering them with distilled water for 2 hours or more. The water is changed three times during the washing and the filters are dried upon a rack.

The Benedict colorimetric procedure was adopted because of its specificity for sugar in the presence of the non-sugar reducing substances of the blood, and also on account of the convenience and practical adaptability of colorimetry to blood analysis. We have tried the zinc and copper deproteinizing methods of Somogyi (9, 10), but, in agreement with Benedict (6), we were not able to

get fermented filtrates free from non-sugar reducing substances when copper reduction methods of determination were used. We also were unable to get quantitative recoveries of galactose added to blood when the zinc deproteinizing method of Somogyi was used, the best recoveries obtained being 80 per cent of added galactose from blood deproteinized by zinc reagents which gave a filtrate with a pH of 7.5. Using the method described above, and deducting the saccharoid value determined by Benedict's (6) method, we obtained recoveries of galactose added to blood ranging from 98 to 108 per cent.

In preparing the standard galactose solution, a stock solution is first made by dissolving 1 gm. of c.p. galactose in 100 cc. of saturated benzoic acid solution. This stock solution is then diluted 100 times with saturated benzoic acid solution to obtain the standard used in blood analysis, which contains 0.1 mg. of galactose per cc. In the absence of adequate information upon the keeping qualities of this standard galactose solution we recommend at this writing that a fresh galactose standard be prepared once in 3 months. Galactose solutions prepared from c.p. Pfanstiehl galactose have 77 per cent of the reducing power of glucose of highest purity (United States Bureau of Standards).

With this method a plus value for galactose is obtained due to the presence of non-sugar reducing substances in blood. The non-sugar, or saccharoid, values of blood obtained by us varied from 4 to 11 mg. per 100 cc. as galactose. Benedict (6) has reported saccharoid values of 5 to 8 mg. per 100 cc. as glucose by his method. The values obtained by us, as galactose, correspond closely to the findings of Benedict, since galactose has only 77 per cent of the reducing power of glucose. One may obtain more specific values for blood galactose by determining the saccharoid content of a control sample of blood collected previous to the administration of galactose and subtracting this value from the results obtained upon blood samples collected later. This should give reliable results as the saccharoid content may not be expected to vary within a few hours. To determine the saccharoid content accurately, the procedure recommended by Benedict (6), in which a known amount of galactose is added to fermented filtrate, must be followed. In the experiments reported in this paper the saccharoid content of a control sample of blood was determined in all cases in

order that specific galactose values might be calculated, and also to have a constant check upon the activity of the yeast used. It makes no practical difference in interpretation of results, however, whether the blood galactose values obtained by this method are corrected for the saccharoid content, or are used uncorrected, since the saccharoid fraction of blood is fairly constant and falls within approximately 10 mg. per cent of the true value when this technique is used.

### *Experimental Procedure*

Our experiments upon human subjects were conducted in the morning, the subjects having fasted since the night before, and no food was allowed during the period of the experiment. A control sample of blood was first obtained. The subjects were given by mouth galactose dissolved in a convenient quantity of water, which was from 300 to 500 cc. Samples of blood were then collected at  $\frac{1}{2}$  hour, 1 hour, and 2 hour intervals following the ingestion of the sugar. The galactose content of the blood samples was determined by the method described above and the total blood sugar was estimated as glucose by the Benedict method (6). One urine sample was collected at the end of the 2 hour period. The galactose content of the urine of the normal subjects, and the amount of both galactose and glucose in the urines of the diabetic subjects, were determined. The amount of galactose given was 1 gm. per kilo of body weight. We believe that in a study in which conclusions are based upon the galactose concentration of the blood, it is very important to establish the galactose dosage according to body weight, because of the variation in the amount of intestinal absorptive surface, the blood volume, and the mass of liver and muscle tissue in subjects of different size. The normal subjects studied were medical students or laboratory workers with no evidences of abnormal conditions. Five male and five female subjects are included in the normal group. The diabetic subjects were patients in an out-patient diabetic clinic. These patients have received treatment for diabetes in this clinic from 1 to 7 years. They include two male and eight female subjects. Seven of the diabetic subjects were also given glucose tolerance tests with the same dosage of glucose as was used for the galactose tests. The glucose tolerance tests were given under conditions



similar to those of the galactose tests and within 1 to 2 weeks following the galactose tests. The galactose used was a C.P. grade of *d*-galactose with a specific rotation of  $+80.5^\circ$ .

### Results

The results of our experiments with normal subjects are shown in Table I. The maximum elevation of the blood galactose occurred in the sample collected 1 hour after ingestion in all cases but one, the latter showing a maximum elevation in the 2 hour sample. The highest elevation of blood galactose in the ten sub-

TABLE I  
*Galactose Tolerance of Normal Subjects*

Subject	Galactose ingested	Blood sugar, mg. per 100 cc.								Urine sugar
		Galactose				Total sugar				Galactose in 2 hr. sample
		Control (saccharoid)	½ hr.	1 hr.	2 hr.	Control	½ hr.	1 hr.	2 hr.	
	gm.									gm.
A.M., ♂	66	8	29	37	32	75	88	95	89	1.00
A.S., ♂	61	7	35	39	36	79	86	90	88	2.25
J.R., ♂	75	9	56	63	33	80	107	118	92	2.48
R.E., ♂	64	9	32	67	30	78	91	112	97	1.00
H.D., ♀	74	7	28	80	34	78	98	118	87	0.91
F.B., ♀	60	10	53	100	29	89	120	142	96	1.35
C.S., ♀	44	7	90	109	34	70	125	139	89	0.92
E.B., ♀	58	9	53	89	118	76	94	132	156	2.20
G.Y., ♀	59	11	80	120	106	80	131	166	151	2.78
G.R., ♂	70	10	67	138	101	73	114	148	138	1.75

jects was 138 mg. per 100 cc.; the lowest was 39 mg. per 100 cc. The total sugar curves parallel the galactose curves in all cases. The 2 hour urine samples all contained galactose, the amounts ranging from 0.91 to 2.78 gm. These results are in general agreement with the work of Harding and van Nostrand (3) who reported studies of the blood and urine galactose concentrations of normal subjects following the ingestion of 50 gm. doses of galactose, except that these authors did not obtain significant elevations of the non-fermentable sugar of the blood in seven of their fourteen subjects; whereas, we obtained definite elevations of the blood

galactose in all of our subjects. There is no evidence in these results of a higher tolerance for galactose in women than in men, as was reported by Rowe (11).

Our results with diabetic subjects are shown in Table II. The first significant observation from these data is that the elevation of the blood galactose following galactose ingestion is no greater in the ten diabetic subjects than that obtained with ten normal subjects. This relation is shown by the curves of Fig. 1. These

TABLE II  
*Galactose Tolerance of Diabetic Subjects*

Subject	Sugar ingested	Galactose ingestion										Glucose ingestion			
		Blood sugar, mg. per 100 cc.										Urine sugar, 2 hr. sample			
		Galactose				Total sugar as glucose						Blood sugar, mg. per 100 cc.			
												Glucose			
		Control (saccharoid)	1 hr.	1 hr.	2 hr.	Control	1 hr.	1 hr.	2 hr.	Galactose	Total sugar as glucose	Control	1 hr.	1 hr.	2 hr.
	gm.									gm.	gm.				
H.C., ♂	50	5	18	20	16	408	416	439	388	0 29	0 23	190	228	266	247
M.J., ♀	50	9	25	47	21	105	114	140	109	0 29	0 23	90	160	220	173
A.S., ♀	60	11	22	77	31	186	242	296	257	0 58	0 46				
S.B., ♀	90	10		25	16	253	250	257	258	1 05	3 19	247	312	402	415
S.T., ♀	62	4	31	59	29	220	276	305	300						
E.L., ♀	60	8	32	52	24	218	280	294	256	1 15	2 14	224	272	356	426
H.Ck., ♀	72	5	18	34	41	179	189	235	247	0 72	1 15	151	240	275	302
M.P., ♀	60	7	51	99	35	205	272	348	305	3 94	8 73	226	330	384	424
H.S., ♂	62	8	52	73	22	410	500	547	500	1 65	10 88				
J.R., ♀	60	8	34	51	25	190	240	270	247	1 39	2 22	188	228	315	346

findings indicate that diabetics have as good a tolerance for galactose as normal subjects, a conclusion that is quite in contrast with the existing impression in the literature. A second significant observation from the data of Table II is that there is no greater excretion of galactose in the urine by the diabetic subjects than by the normal subjects. A third fact of importance is that the total sugar values of the blood parallel the galactose findings, but in some cases there is a greater increase in total sugar than in galactose. This relation is shown in Fig. 2. The curves showing

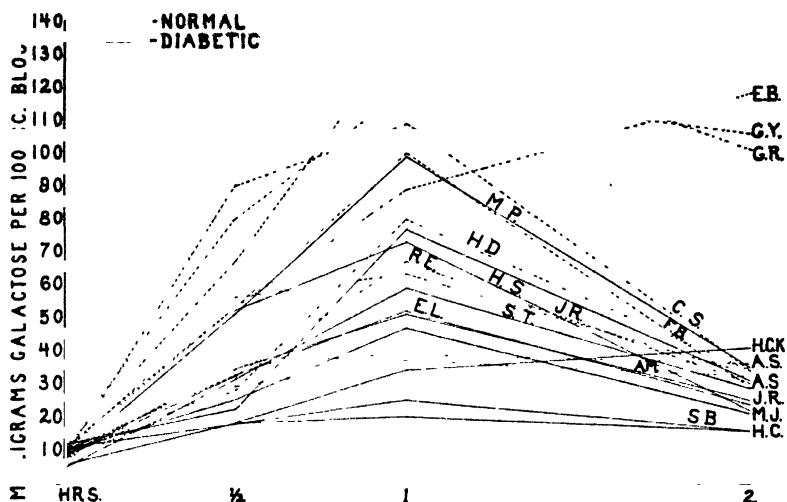


FIG. 1. Blood galactose curves following the ingestion of 1 gm. of galactose per kilo of body weight for ten normal and ten diabetic subjects.

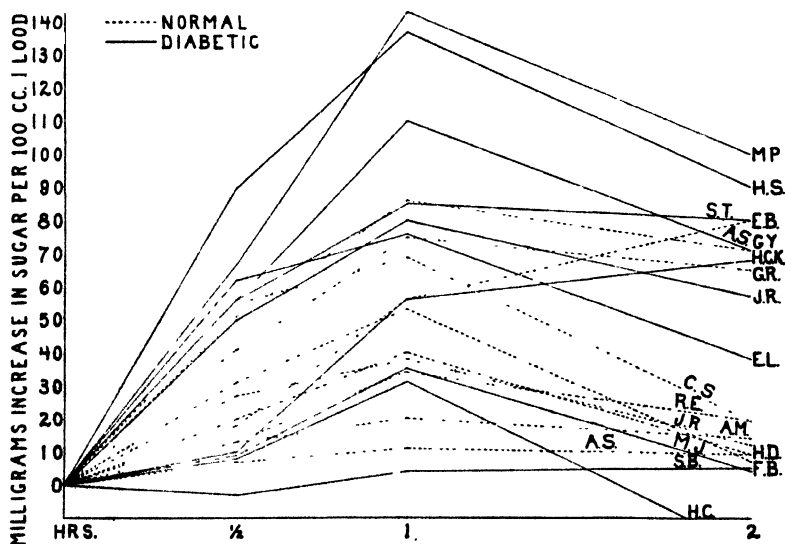


FIG. 2. Curves showing elevation of total blood sugar following the ingestion of 1 gm. of galactose per kilo of body weight for ten normal and ten diabetic subjects.

the elevation of the total blood sugar for seven of the diabetic subjects are well within the limits of the elevation of the total sugar curves of the normal subjects, but the curves for three diabetic subjects show a greater elevation than those of the normals. The greater increase in total blood sugar than in blood galactose following galactose ingestion is apparently due to conversion of galactose into fermentable sugar. This does not alter appreciably the demonstration that diabetics have good tolerance for galactose, because the elevations of total blood sugar were not marked in the three subjects showing greater increases in total sugar than was obtained with normal subjects. The evidence for conversion of galactose to fermentable sugar will be discussed later in this paper.

The observation that diabetics metabolize galactose practically as satisfactorily as normal subjects suggested to us the desirability of demonstrating the presence and the severity of the diabetes in these subjects by means of glucose tolerance tests, and of obtaining a comparison of the tolerance of diabetics for these two sugars. Accordingly glucose tolerance tests were given to seven of the diabetic subjects within 1 to 2 weeks following the galactose tolerance test, the condition of these subjects being unchanged at the time of the glucose test in so far as revealed by clinical observation and the blood sugar concentration. The amount of glucose administered was the same as the amount of galactose given in the galactose tolerance tests. The results of the glucose tolerance tests are shown in Table II. A characteristic diabetic elevation of the blood sugar was obtained in all subjects. The relation between the responses from galactose ingestion and glucose ingestion is shown in Fig. 3. In preparing these curves it was necessary to add a plus correction to the total sugar values obtained by galactose ingestion because the total sugar of the blood following galactose ingestion is a mixture consisting of both glucose and galactose. In determining these mixed sugars by Benedict's method, in which a glucose standard is used, the glucose is estimated correctly, but the galactose is underestimated because it has only 77 per cent of the reducing power of glucose. To correct for this underestimation of galactose it is necessary to add to the total sugar 23 per cent of the galactose value obtained in the separate estimation of the blood galactose. A correction calcu-

lated in this manner was added to the total sugar values obtained after galactose ingestion and these values were used for plotting the curves representing galactose tolerance in Fig. 3. To simplify the comparison the galactose tolerance and glucose tolerance curves are started at the same origin. The comparison shown by these curves is a striking one. There is a marked spread between the two curves, and in all cases but one, the glucose tolerance

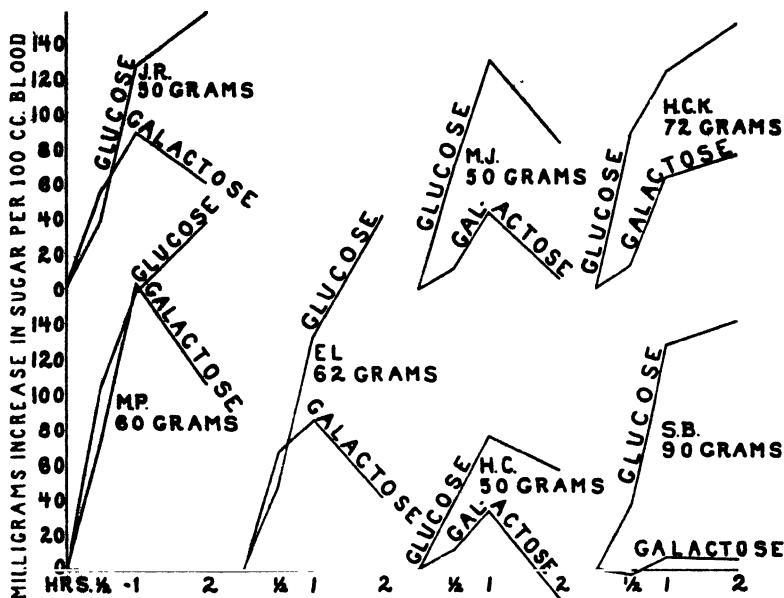


FIG. 3. Comparison of galactose and glucose tolerance of seven diabetic subjects, the dosage of galactose and glucose being the same in each case. The upper of each pair of curves shows the response following ingestion of glucose; the lower shows the results following the ingestion of galactose.

curves show maximum elevations at the end of the 2 hour period, while the galactose tolerance curves show a marked lowering at the end of the 2 hour period in all but one instance. These findings demonstrate clearly the greater tolerance of diabetics for galactose than for glucose.

In seeking an explanation of the practically normal tolerance of diabetics for galactose a negative relationship of insulin to galactose metabolism seemed probable. This conception is con-

trary to the existing opinion and evidence in the literature regarding the activity of insulin with respect to galactose. Corley (1) reported that insulin brought about a marked lowering of galactose in the blood of a rabbit when 1 gm. of galactose and 2 units of insulin in 15 cc. of water were injected intravenously. The uncontrolled experiment reported by Corley does not justify the conclusion he made. Just because the galactose content of the blood of a rabbit was lowered following insulin administration is no reason to assume that insulin is responsible for this lowering.

To examine the relation of insulin to galactose metabolism certain animal experiments were carried out. In our first experiments upon this problem mixtures of equal parts of galactose and glucose were given intravenously, or subcutaneously, and insulin, in doses calculated to be a little less than lethal for the amount of glucose administered, was injected subcutaneously. Samples of blood were then collected at intervals following administration of the sugars and the total blood sugar and blood galactose were determined upon these samples. In such experiments the total sugar curves may be interpreted as showing the effect of insulin upon glucose, since a considerable part of the total sugar under these conditions is glucose; and the galactose curves should represent the influence of insulin upon galactose in the blood. The value of such experiments is that they show the simultaneous effects of insulin in the same animal upon comparable amounts of circulating glucose and galactose. Typical results of such experiments upon rabbits and upon chickens are shown in Fig. 4. The slopes of the curves in Fig. 4 show a more rapid lowering of the total blood sugar than of the blood galactose. This effect continues until nearly all of the glucose has disappeared from the blood, at which time the total sugar curve and the galactose curve either cross or parallel each other. These experiments demonstrate that the effect of insulin upon glucose is certainly more marked than its influence, if any, upon galactose.

Further experiments upon this problem consisted of studying the rate of removal of injected galactose from the blood of rabbits with and without insulin administration. Two rabbits were given 2 gm. of galactose per kilo of body weight intraperitoneally, and, after waiting about 1 hour to permit the blood galactose to reach a peak level, samples of blood were collected at approxi-

mately  $\frac{1}{2}$  hour intervals until 3 hours after injection of the sugar. In this way control values upon the rate of removal of galactose

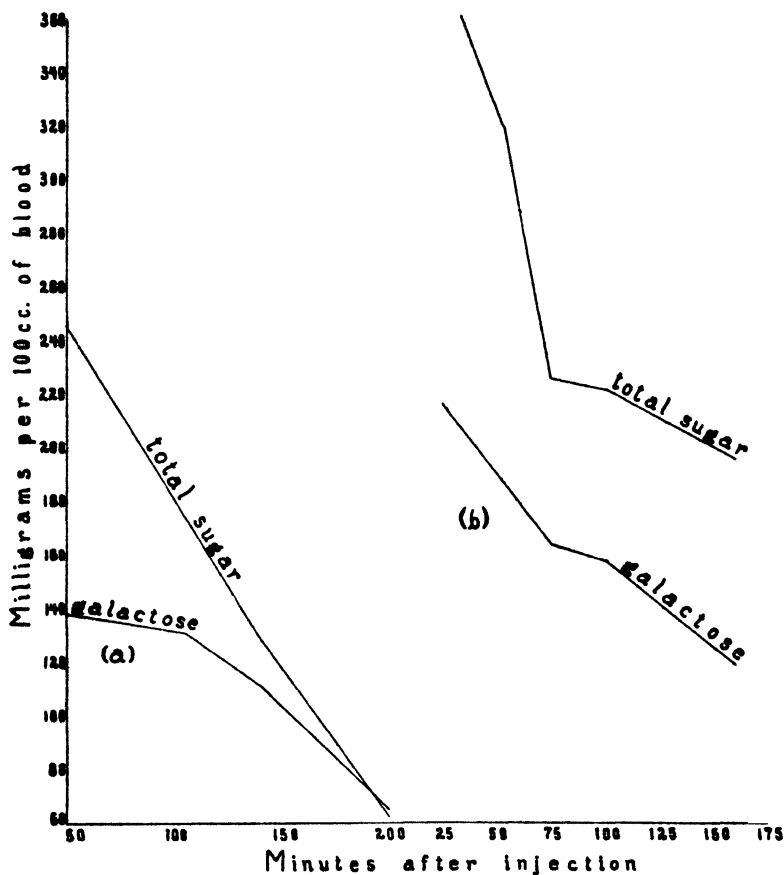


FIG. 4. Total blood sugar and blood galactose curves following the administration of 2 gm. of glucose, 2 gm. of galactose, and 4 units of insulin per kilo of body weight to a rabbit (a) and 2 gm. of glucose, 2 gm. of galactose, and 2 units of insulin per kilo of body weight to a chicken (b). The sugar was given to the rabbit subcutaneously and to the chicken intravenously.

from the blood were obtained. Upon the following day this experimental procedure was repeated with the additional step of injecting 9 clinical units of insulin following the galactose adminis-

tration. Care was taken to inject the same dosage of galactose in the same manner and blood samples were collected at intervals corresponding to those of the control procedure. The results of these experiments are shown in Fig. 5. The curves obtained with insulin administration show a little less drop in the blood galactose

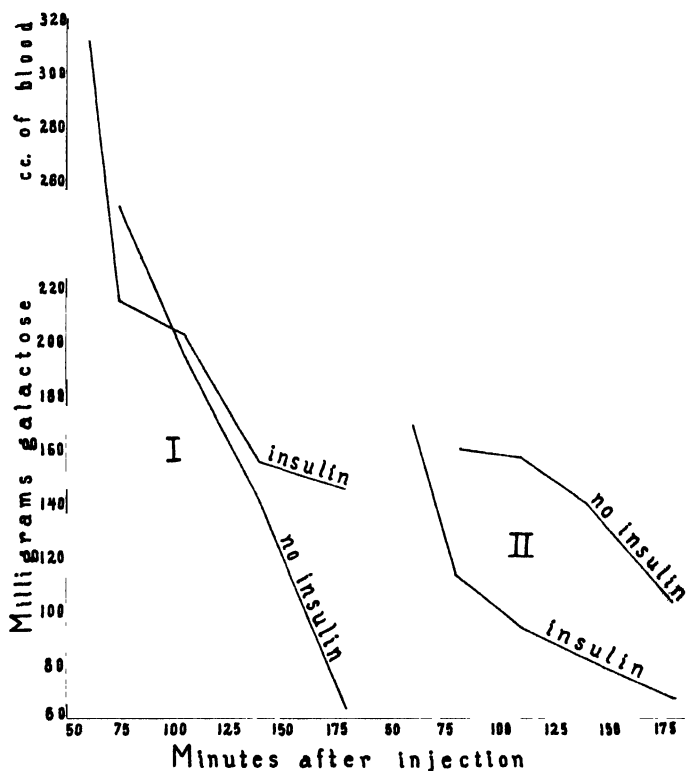


FIG. 5. Blood galactose curves obtained upon rabbits given 2 gm. of galactose per kilo of body weight with and without insulin administration.

than was obtained over the corresponding period in the control procedure in which insulin was not injected. To make sure that the insulin was potent the rabbits were observed until insulin convulsions occurred, and blood samples were collected at the stage of convulsions and analyzed for fermentable sugar, the results of these analyses showing that a hypoglycemia existed. The



failure of toxic doses of insulin to influence the removal of galactose from the blood of rabbits, as shown by these experiments, is convincing evidence that insulin does not affect the anabolism of galactose.

To study further the relation of insulin to galactose metabolism it was decided to investigate the ability of galactose to detoxify lethal doses of insulin. The experiments carried out by us consisted of giving large doses of galactose subcutaneously to rabbits, followed by injection of about 3 times the lethal dose of insulin. The results of these experiments are shown in Table III. In three experiments 10 gm. of galactose failed to prevent the onset of insulin convulsions when 10 clinical units of insulin were injected, and in one experiment 20 gm. of galactose did not prevent convulsions, the insulin dosage being 15 clinical units. A sample

TABLE III  
*Showing Galactose Is Not a Physiological Antagonist to Insulin*

Rabbit No.	Clinical units of insulin injected	Galactose injected	Blood galactose at time of convulsions
		gm.	mg. per 100 cc.
I	10	10	150
II	10	10	254
III	10	10	270
IV	15	20	409

of blood was collected from the marginal ear vein of the rabbits after convulsions had occurred and the animal was in collapse, and these samples were analyzed for galactose by our method and for total sugar by Benedict's method. The total sugar values were the same within the limits of experimental error before and after yeast fermentation, showing that the glucose had practically all disappeared. The analyses for galactose showed values ranging from 150 to 409 mg. of galactose per 100 cc. of blood. The onset of insulin convulsions following insulin and galactose administration, and the demonstration of large amounts of galactose in blood collected during the stage of insulin convulsions, are indisputable proof that galactose is not a direct physiological antagonist to insulin. This finding is also further evidence that insulin does not influence the anabolism of galactose.

The power of galactose to detoxify insulin has been studied by other workers. Noble and Macleod (12) have reported that galactose injected subcutaneously has a slight antidote effect upon insulin but does not prevent insulin convulsions in rabbits given toxic doses of this hormone. Voegtlin, Dunn, and Thompson (13) found that galactose has almost as good a protective influence against minimum lethal doses of insulin administered to rats as has glucose, when the sugar is given by way of the alimentary tract with a stomach tube. Our work on the other hand has shown that galactose is not a physiological antagonist to insulin. These reports may be harmonized by assuming that following galactose administration there is some conversion of galactose into a sugar that has an antidote effect upon insulin. The conversion of galactose into a sugar that is a specific antidote for insulin would account for the slight detoxifying effects obtained by Noble and Macleod by subcutaneous injection of galactose, and would explain the more marked detoxifying effects obtained by Voegtlin, Dunn, and Thompson as there is probably more conversion when the sugar is administered by way of the alimentary tract. Such a hypothesis is in harmony with our finding that galactose *per se* is not an antidote for insulin. Later in this paper we will discuss other evidence for the conversion of galactose into fermentable sugar following galactose ingestion.

In the experiments described above it has been shown that (1) insulin injected into animals following the administration of equal quantities of glucose and galactose has a marked effect upon the lowering of the blood glucose and that its influence, if any, upon the blood galactose is uncertain; that (2) toxic doses of insulin, when administered to rabbits which have received galactose intraperitoneally, did not cause a more marked lowering of the blood galactose than was obtained in control experiments in which the rabbits were given the same dosage of galactose but did not receive insulin; that (3) galactose is not a physiological antagonist to insulin. These findings are convincing evidence that insulin does not influence the anabolism of galactose, a conclusion which is in harmony with our observation that the removal of galactose from the blood is as rapid in diabetic subjects as in normal subjects.

*Is Galactose Converted to Fermentable Sugar during or following Absorption?*

The data of our experiments with human subjects have a bearing upon the question of whether galactose is converted to fermentable sugar during or following absorption from the alimentary tract. If the increase in total blood sugar following ingestion of galactose is greater than the increase in blood galactose, there is either conversion of galactose into fermentable sugar, or else a physiological stimulus bringing about increased glycogenolysis has occurred. Our experiments yielded data which are capable of analysis for increases in blood galactose and total blood sugar. To get the increase in blood galactose we subtract the saccharoid value of a control sample of blood obtained before galactose ingestion from the galactose values of samples of blood collected after galactose ingestion. To calculate the increase in total blood sugar we first correct the total sugar values by adding 23 per cent of the galactose found in the same sample of blood by the separate analysis for galactose; this correction is valid, because the galactose fraction of the total blood sugar was underestimated 23 per cent by being determined against a glucose standard. From the corrected total blood sugar values we then subtract the initial total sugar value obtained upon a control sample of blood collected before ingestion of galactose; this gives the increase in total blood sugar following galactose ingestion. The analysis of our data is given in Table IV. In this table is shown the difference between the total blood sugar increase and the blood galactose increase, expressed as increase in fermentable sugar, in the  $\frac{1}{2}$  hour, 1 hour, and 2 hour samples of blood collected following galactose ingestion in both the normal and diabetic subjects. A definite contrast in findings occurs. In the normal subjects slightly negative values for fermentable sugar are obtained in nearly all cases; in the diabetic subjects positive values for fermentable sugar that represent increases beyond the limits of experimental error are the rule. The positive values for increases in fermentable sugar obtained upon diabetic subjects must be interpreted as indicating that either there was a conversion of galactose into fermentable sugar or that increased glycogenolysis occurred. That increased glycogenolysis occurred in the diabetic subjects following galactose ingestion seems very

improbable because the increase in the monosaccharide content of the blood by absorption from the intestinal tract would certainly oppose the glycogenolysis reaction. It seems probable, therefore, from our experiments, that in diabetic subjects there is a small amount of conversion of galactose into fermentable sugar following galactose ingestion, either during absorption from the intestinal tract or while the galactose is present in the blood. This conclusion is in harmony with the fact that diabetic subjects store glucose poorly, and hence, if conversion of galactose occurs,

TABLE IV

*Difference between Total Sugar Increase and Galactose Increase in Blood Following Galactose Ingestion*

Calculated from the data of Tables I and II.

Normal subjects				Diabetic subjects			
Subject	Fermentable sugar per 100 cc.			Subject	Fermentable sugar per 100 cc.		
	½ hr. sample	1 hr. sample	2 hr. sample		½ hr. sample	1 hr. sample	2 hr. sample
	mg.	mg.	mg.		mg.	mg.	mg.
A.M.	-3	-2	-5	H.C.	-2	+19	
A.S.	-15	-14	-14	M.J.	-4	+6	-6
J.R.	-9	-5	-6	A.S.	+47	+59	+56
R.E.	-5	-11	+3	S.B.		-8	0
H.D.	+4	-16	-12	S.T.	+35	+43	+61
F.B.	-3	-16	-8	E.L.	+43	+42	+25
C.S.	-9	-10	-2	H.Ck.	0	+34	+40
E.B.	-16	-6	-4	M.P.	+32	+72	+78
G.Y.	-2	+2	-2	H.S.	+56	+87	+79
G.R.	+5	-24	-5	J.R.	+30	+47	+44

fermentable sugar will tend to accumulate in the blood. Our data upon normal subjects do not give evidence either for, or against, conversion of galactose into fermentable sugar following galactose ingestion, yet some conversion may have occurred, because normal subjects store glucose rapidly and possibly with increases in blood galactose the mechanism is to lower the blood sugar by storage of glucose. Such a mechanism would account for the negative values for increases in fermentable sugar in the blood of our normal subjects following galactose ingestion.

*Utilization of Galactose*

Harding and van Nostrand (3) have studied the utilization of galactose and have corrected the impression in the literature that galactose is a poorly utilized sugar. The idea that galactose is poorly utilized arose from qualitative studies of the urine following galactose administration. By means of quantitative studies of urinary galactose excretion Harding and van Nostrand showed that galactose is well utilized by the human body. These authors gave 50 gm. of galactose by mouth and found a utilization of 97 per cent in half of their subjects, as measured by urinary galactose excretion. The poorest utilization observed by Harding and van Nostrand was 86 per cent of ingested galactose. We cannot draw conclusions regarding complete utilization of galactose from our data, because in our experiments only 2 hour quantities of urine were collected, and galactose is excreted in the urine for a longer time than 2 hours after ingestion of such doses as we used. Our studies of urinary galactose excretion were carried out to obtain a comparison of the utilization of galactose by normal and diabetic subjects. Our results show no greater excretion of galactose in the urine by the diabetic subjects than by the normal subjects. Two of our diabetic subjects, M. J. and A. S., showed a low galactose excretion and no glucose excretion. In the other diabetic subjects there was both glucose and galactose excretion in the urine. The glucose excretion in these cases was no greater than would seem to be warranted by the degree of hyperglycemia that existed. The results of our comparative urinary sugar excretion studies indicate that the utilization of galactose by diabetics is as good as its utilization by normal subjects.

*Clinical Import*

From our experiments certain clinical possibilities are suggested. Our data show that galactose is well tolerated by diabetics when given in large doses. The administration of galactose in smaller amounts along with other foods should result in even better tolerance and less urinary excretion. It, therefore, seems probable that galactose might be made a valuable adjunct to the diet in the clinical management of diabetes mellitus. The use of a

sugar that has nutritive value and a satisfying sweetness, and whose administration by mouth produces practically no greater saccharemia in diabetics than in normal subjects, should mark considerable advance in the control of diabetes mellitus and its undesirable consequences, such as diabetic retinitis and endarteritis, conditions which are presumably etiologically related to hyperglycemia. Our work has demonstrated that galactose ingestion by the diabetic does not produce the hypersaccharemia characteristic of glucose ingestion, and that this is apparently because insulin is not necessary for the anabolism of galactose. Deuel, Gulick, and Butts (14) have reported that galactose has a higher antiketogenic value than glucose, an observation that suggests further possibilities from galactose feeding to diabetic patients. However, a complete evaluation of the possibilities of galactose feeding in diabetes mellitus is conditioned upon further knowledge of the metabolism of galactose, and of the part played by insulin in carbohydrate catabolism.

#### SUMMARY

1. A method for the determination of galactose in blood is described.
2. The galactose tolerance of ten normal and ten diabetic subjects has been studied by the determination of the blood galactose, total blood sugar, and urinary galactose, following the ingestion of 1 gm. of galactose per kilo of body weight.
3. The blood galactose values following galactose ingestion obtained with diabetic subjects were in all cases within the limits of the values obtained with normal subjects upon the same galactose dosage.
4. The total blood sugar increases following galactose ingestion were no greater in seven of the diabetic subjects than the total blood sugar increases obtained with normal subjects. The other three diabetic subjects showed slightly greater total blood sugar increases following galactose ingestion than was obtained with normal subjects, a result which is apparently due to the conversion of galactose to fermentable sugar.
5. It has been shown that toxic doses of insulin do not change the rate of removal of galactose from the blood of rabbits and that galactose is not a direct physiological antagonist to insulin. It

seems evident, therefore, that insulin does not influence the anabolism of galactose. This conclusion offers a satisfactory explanation of our data showing that diabetics have practically as good a tolerance for galactose as normal subjects.

6. Clinical possibilities of the use of galactose in the control of diabetes mellitus are suggested.

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## FAT TRANSPORT THROUGH THE LYMPH SYSTEM IN FASTING AND PHLORHIZIN POISONING

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It is an old observation in physiology that ingested fat after absorption by the bowel is transported through the thoracic duct into the blood. The transport of body fat in hunger, certain experimental poisonings, and toxemias is thought to be effected by the blood system, the fat of the fat depots being taken up directly into the capillary blood vessels. The efforts of many investigators to study the transport of body fat in experimental conditions by analysis of the blood fat have yielded few conclusive facts, although the methods have been improved considerably. In fasting, for instance, when body fat is presumably transported through the blood system definite changes in the fat content of the blood cannot be demonstrated. Leathes and Raper (1) explain this by stating that "active transportation of fat in the blood need not mean an increased amount of fat in the blood at any moment," since "the amount of fat in the blood depends necessarily on two factors: the rate at which additional fat enters and the rate at which it leaves." The faster the reserve fat is thrown into the blood, the faster it will be removed by the hungry cells; this regulation seems to be sufficiently delicate to prevent marked changes in the blood fat content. The fact that in fasting the mobilized reserve fat cannot be demonstrated in the blood might be explained, however, on a different assumption. It has been claimed recently by several investigators, Hoffmann and Wertheimer (2) and Schur and Löw (3), that the reserve fat in fasting is not conveyed as such at all, but that it is transformed into glycogen and sugar in the fat depots and that this sugar is then transported by the blood to the tissue cells.

It occurred to us that if reserve fat when mobilized in fasting is transported as fat, it might be carried to some extent at least by the lymph system. If this assumption is correct, it should be easy to demonstrate considerable fat in the lymph obtained from the thoracic duct of a fasting animal, since the fat content of this lymph would depend only on the rate of the fat mobilization, the absorption of this fat by the hungry tissues being entirely eliminated.

#### EXPERIMENTAL

In one series of experiments, dogs which had been fasted from 36 hours to 14 days were used. In the second series, the fasting animals were poisoned with phlorhizin. 1.5 to 3.0 gm. of phlorhizin dissolved in 15 to 30 cc. of warm 2 per cent  $\text{NaHCO}_3$  were injected subcutaneously on the 3rd and 4th days of fasting, and the dog was operated on on the 5th day. In the early experiments, morphine and ether were used for anesthesia, but were later replaced by nembutal (Abbott), 30 mg. per kilo intravenously, which served very satisfactorily. The thoracic duct was cannulated and samples of lymph were collected in tubes containing potassium oxalate. In some experiments, a cervical lymph vessel was also cannulated and cervical lymph collected. Venous blood was drawn at the same time. The lymph and blood plasma were analyzed for total fatty acids and cholesterol by Bloor's oxidation-titration method (4). Sugar was determined in the oxalated blood and lymph by the Folin (5) modification of the Folin-Wu sugar method. Chylomicron counts were made on the lymph and blood, the dark-field microscope being used in the manner described by Gage and Fish (6).

#### Results

*Fasting*—The lymph obtained in all cases was more or less opalescent and contained a few lymphocytes. The chylomicron count of the lymph varied between 75 and 200, while the blood at the same time showed never more than 1 or 2.

As is evident from Table I fasting lymph always contains fat, sometimes in quite considerable amounts. It appears that the amount of fat contained in fasting lymph depends only partly on the duration of the fast, the state of nutrition and the age of the animal being, among other factors, instrumental.

Our finding that the lymph contains considerable fat even on the 2nd day of fasting seems to be at variance with statements of earlier investigators (7), that 24 hours after a fat meal the thoracic duct lymph contains only traces of fat. However, in this early work the fat was determined by extracting the dried lymph with ether. It is known (8) that in body fluids containing large amounts of protein only part of the total fat—the “free” fat—is

TABLE I  
*Fatty Acids, Cholesterol, and Sugar in Lymph and Blood of Fasted Dogs*

Dog No.	Days fasted	Lymph			Blood		
		Total fatty acids	Cholesterol	Sugar	Total fatty acids	Cholesterol	Sugar
		mg. per 100 cc.	mg per 100 cc.	mg per 100 cc.	mg. per 100 cc.	mg per 100 cc.	mg. per 100 cc.
80	1 5	332	61	59 5	277	116	125
83	1 5	385	114	42 6			95.2
78	1 5	199*	55*				
89	2	454	55				
91	3	514	69				
62†	4	280	78				
64†	4	269	68				
65†	4	270	77				
87	4	249	69	54			
76	7	482	77	38.4	157	66	55 5
97	8	270	83				
		110*	39*				
72	9	1030	66	59	332	69	67
74	9	415	61	35 7	285	86	57.1
79	9	609	69				
92	11	645	75		371	91	
93	14	415	41		360	55	

\* Cervical lymph.

† Ether anesthesia.

determined this way; the alcohol-ether extraction in Bloor's method brings out the fat combined with protein also.

The marked opalescence of fasting lymph and the high number of chylomicrons present suggest that a considerable part of the fat might be in the “free” form. Analysis showed that the fat extracted from dried lymph of fasting animals by ether amounts to about one-half of the total fat extractable by alcohol-ether (in one

case 290 mg. as against 540 mg.). No efforts were made at this time to identify the individual lipids or fatty acids and their proportions in the lymph.

The fat content of fasting lymph is usually higher than that of the blood drawn at the same time. The cholesterol content is less, occasionally much less, than that of the corresponding blood. The ratio cholesterol : fatty acids, varies from 1:3 to 1:15 in the fasting lymph, and from 1:2 to 1:6 in the fasting blood.

The glucose content of fasting lymph is always considerably less than that of the corresponding blood. It is also less than in

TABLE II  
*Fatty Acids, Cholesterol, and Sugar in Lymph and Blood of Dogs 1 Day after Phlorhizin Injection*

Dog No.	Lymph			Blood		
	Total fatty acids	Cholesterol	Sugar	Total fatty acids	Cholesterol	Sugar
	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.
58*	1140	138				
61*	692	98		360	169	
67	720	105		352	171	
70	756	98	23	560	270	36
71	412	74		609	140	
73	706	83	24	499	138	50
75	715	44	25	426	98	41.7
	878†	58†	27†			

\* Ether anesthesia.

† Cervical lymph.

normal lymph, which is known to contain as much sugar as the blood (9).

*Phlorhizin Poisoning*—All animals exhibited marked glycosuria on the day of the operation. The lymph in these cases was usually more opalescent than the fasting lymph, and sometimes resembled skim milk. The cervical lymph obtained in one case, Dog 75, was also quite milky. The sediment and chylomicron counts of the phlorhizin lymph were similar to those of the fasting lymph.

The data in Table II show that the lymph in phlorhizin poison-

ing contains considerable amounts of fat, usually more than during fasting of similar duration. The cervical lymph contained even more fat than the corresponding thoracic duct lymph. With one exception, Dog 71, the fat content of the lymph was higher than that of the corresponding blood.

The fat content of the blood was rather high in three of the phlorhizin-injected dogs, Dogs 70, 71, 73; in the other three, it was within normal limits although higher than in most of our fasting dogs. The cholesterol content of the lymph was in each case much less than that of the corresponding blood, but usually higher than that of fasting lymph. Most of the phlorhizin-injected dogs exhibited hypercholesterolemia. The ratio, cholesterol : fatty acids, varied from 1:6 to 1:16 in the phlorhizin lymph, and from 1:2 to 1:4 in the blood.

The sugar content of the phlorhizin blood was low, but that of the phlorhizin lymph was still lower.

#### DISCUSSION

The possibility of the presence in the lymph of substances other than fatty acids of high molecular weight titratable by Bloor's method must be considered. Lactic acid and especially  $\beta$ -hydroxybutyric acid might be present in the lymph under the experimental conditions. However, they are soluble in water and practically insoluble in petroleum ether, and would therefore not appear in the final extractions. Acetone and diacetic acid, if present, would be entirely eliminated during the repeated processes of evaporation. The anesthetic used, nembutal (pentobarbital sodium), is insoluble in petroleum ether. Qualitative tests easily demonstrated the presence of fat in the lymph.

Where does the fat found in the lymph during fasting come from? It might be blood fat filtered through the blood capillaries directly into the lymph spaces and lymph vessels. However, the fact that the lymph usually contains much more fat than the blood, while at the same time it always contains much less sugar and cholesterol, indicates that at least part of the lymph fat must come from sources other than the blood. Another suggestive evidence of this is our finding of chylomicrons in the lymph in fasting, with none in the blood.

To settle this point definitely, we examined the fat content of cervical lymph at the height of alimentary lipemia. After 24 hours fasting, two dogs were fed each  $\frac{1}{2}$  pint of cream and four egg yolks. 4 hours later the cervical lymph duct was cannulated and lymph collected for 1 hour. Immediately following this, the thoracic duct was cannulated. The clear cervical lymph contained 200 mg. and 210 mg. of fat per 100 cc., respectively, while the milky thoracic duct chyle contained 1580 mg. and 4500 mg. of fat per 100 cc. In these dogs evidently there was movement of considerable fat through the thoracic duct into the blood and from the blood into the tissues; yet the cervical lymph at the same time contained very little fat. This shows clearly that blood fat does not directly enter the lymphatics even when it leaves the blood stream rapidly and in large amounts.

We must conclude then that the lymph fat in fasting comes chiefly from the reserve fat of the depots and tissues. We have not as yet attempted to determine which tissues are the chief sources of this mobilized fat. However, it is evident from our data that the liver and other abdominal organs are not exclusive sources since the cervical lymph may also contain considerable fat.

What is the physiological importance of the fat transport through the lymph system in fasting? On calculating the 24 hour amount of the lymph flow in fasting at about one-sixteenth (this is an estimate from lymph fistula dogs) of the body weight (10), it is evident that the total amount of fat transported this way is but a fraction of the total fat consumption, probably not more than 20 per cent. The remainder of the mobilized fat is then either taken up by the blood capillaries of the fat depots and transported by the blood system directly, or it is transformed in the fat depots into sugar and transported as sugar. It is also possible that both these processes are taking place at the same time. However, our finding that both in fasting and phlorhizin poisoning, the lymph contains less *sugar* than normally, speaks strongly against the transport of mobilized fat in the form of sugar. Accordingly, most of the reserve fat must enter the blood capillaries, at a rate proportional to that at which it enters the lymph system.

We believe that the fat content of fasting lymph offers a reliable index of fat mobilization. It is possible to study the rate of fat

mobilization under various experimental conditions by analyzing the fat content of the lymph.

#### SUMMARY AND CONCLUSIONS

1. The lymph contains considerable amounts of fat in fasting and in phlorhizin poisoning. There is more fat but less cholesterol than in the corresponding blood.

2. The sugar content of fasting and phlorhizin lymph is always considerably less than that of the corresponding blood, and also less than that of normal lymph. This finding speaks strongly against the transport of mobilized fat in the form of sugar.

3. Most of the fat in fasting lymph is mobilized reserve fat.

4. Fat transport through the lymph system offers new possibilities to study the phenomena of fat mobilization.

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## STUDIES ON RENNIN

### I. THE PURIFICATION OF RENNIN AND ITS SEPARATION FROM PEPSIN\*

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#### INTRODUCTION

Hammarsten's view that rennin and pepsin are separate enzymes has been challenged by the Pavlov school and others, such as Nencki and Sieber and Pekelharing (1), who maintain the existence of a mammoth molecule with side chains having a proteolytic action in acid media and a rennet action in neutral media. According to this view peptic and rennet activity are due to the same enzyme. Their standpoint has been supported by the fact that proteases, wherever found in nature, are able to clot milk.

Recently Lüers and Bader (2) attempted the purification of rennin according to one of the new methods of enzyme chemistry; *i.e.*, adsorption with aluminum hydroxide. They described a rennin preparation nearly free of protein, giving slight biuret and xanthoproteic reactions, but negative to the ninhydrin and Millon tests. The salt-free dry substance had a nitrogen content of only 0.678 per cent. According to these authors, their preparation was extremely active. The rennet activity increased 39 times and the peptic activity 21 times when compared with the liquid commercial starting material. Lüers and Bader say that, due to experimental difficulties and complications in the method of purification, the difference in activity is too small to allow a decision in favor of two distinct enzymes, rennin and pepsin. In

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this connection, the early work of Sundberg (3) and of Schrumph (4) on the preparation of protein-free pepsin may be noted.

Of the great number of papers dealing with the purification of rennin, Fenger's (5) recent one is most interesting. He obtained very active preparations by extraction of the dry mucosa with  $H_2O$  and  $HCl$  and repeated precipitation with alkali. As the purity of the various fractions increased they lost in peptic activity and increased in rennet activity. However, he was unable to obtain rennin completely free of peptic activity, although he very nearly accomplished that aim.

Because of the recent isolation of pepsin in crystalline form by Northrop (6), and since none of the recorded findings gives evidence of a distinct enzyme, rennin, we were impelled to try to isolate rennin by crystallization or other means, for a comparative study with crystalline pepsin. Theoretically every pure substance should crystallize, but our efforts to crystallize rennin were not realized. However, highly active fractions were obtained in these first trials, which are briefly described as follows:

#### *Preliminary Experiments*

Highly concentrated rennin solutions from which microscopic globular bodies separated out were prepared as follows: To 100 gm. of a special concentrated dry rennin, furnished us by Chr. Hansen's Laboratory, Inc., 100 cc. of  $H_2O$  and 100 cc. of  $\frac{2}{3} N H_2SO_4$  were added. This suspension was dialyzed until free of salt and then  $\frac{2}{3} N H_2SO_4$  was added to effect solution. After filtration,  $MgSO_4$  was added to saturation and the precipitate was centrifuged off. This precipitate was rubbed up to a paste with  $H_2O$  and then treated with  $0.1 N NaOH$  until it dissolved. Additional  $0.1 N NaOH$  was added until the solution became slightly turbid. On standing overnight at room temperature a minute quantity of highly refractive globules separated out and a solution of them had an exceedingly high rennet activity. Careful study was impossible and this method was abandoned. We now attempted to purify the enzyme by other methods, centering our attention upon the relative rennet and peptic activities. By a combination of isoelectric and fractional precipitation we have obtained a preparation in which rennin is extremely active but with no peptic power.

Since all of the commercial preparations we used contained more

or less insoluble material and most of them a difficultly separable "base," and since their origin and method of manufacture is secret, we decided to prepare purified rennin directly from the fourth stomach of the calf.

The following, we believe to be the important factors in purifying rennin: speed, simplicity, the avoidance of alkali, to which this enzyme is very sensitive, and the avoidance of inorganic salts, which are nearly inseparable from the rennin. While working on the commercial preparations referred to above, we found that a highly active fraction could be obtained by making the slightly acid solution of the NaCl-free dialyzed rennin 50 per cent alcoholic and adding NaOH to pH 5.4. The precipitate which formed at this point was many times more active than the original substance or than the subsequent fractions obtained by addition of more alcohol. The fraction precipitated at pH 5.4 had also very little peptic activity. Pepsin does not precipitate at this pH when treated similarly, its isoelectric point being 2.75.

#### *Preparation of Purified Rennin from the Fourth Stomach of the Calf*

Fourth stomachs of the calf were obtained early in the morning from the slaughter-house, immediately after death of the animals. The stomachs were washed in cold water. In the laboratory they were brushed and washed again with cold water to free them from mucin. The mucosa then was dissected from the muscular wall and from fat. The pyloric end was discarded since it was found to contain little rennin and much mucin. The mucosa was minced as finely as possible. About 1 kilo of minced mucosa can be obtained from twelve calf stomachs. To each kilo of ground mucosa 1000 cc. of 0.04 N HCl were added and the mixture stirred with a glass rod for 8 minutes. The dilute HCl extracts the rennin, but the mixing should not be continued for more than 8 minutes since the activity of the final product will be lessened because of the increased amount of inert material extracted and because too much of the liquid will be absorbed by the ground mucosa. The mixture is quickly squeezed through towelling and must then be filtered through filter paper. The clear, slightly pink filtrate should have a pH of about 5.2. If 1 kilo of mucosa is used, about 700 cc. will be obtained. It is then dialyzed in a collodion sac against 3 liters of distilled water, which is frequently changed, until the pH is about

5.4; this requires 3 to 4 hours of dialysis. After this, the solution is transferred to a large beaker, stirred with a rod, and 95 per cent alcohol added slowly to make the extract 50 per cent alcoholic. A fine precipitate forms which after a few minutes standing increases in size and can be centrifuged. We usually filter off two-thirds of the solution rapidly, and then transfer it to 50 cc. centrifuge tubes. The white precipitate is dissolved in 150 cc. of distilled water at room temperature by stirring and centrifuged again to free it from mucin and other insoluble matter. To the opalescent solution 95 per cent ethyl alcohol is added to make the solution 50 per cent alcoholic. The rennin precipitates in white flocculent particles. This, when dissolved in 0.04 N HCl and diluted, shows an enormous activity, greater than that of any hitherto reported pepsin-free preparation, and increases in potency when the above procedure is repeated, of course with some loss of active material. The precipitated rennin can be dried at room temperature *in vacuo* over sulfuric acid, although drying inactivates about half of the enzyme. Since alcohol, if left too long in contact with the rennin, destroys a great part of it, it is advisable to start the drying process the same day. The yield is about 1 gm. of dry substance from 1 kilo of mucosa.

#### *General Properties and Analysis of Most Active Preparation*

The vacuum-dried preparation has a yellowish color and is inactivated on heating. It differs from crystalline pepsin as follows: It is very easily and completely soluble in slightly acidified water, as *e.g.* 0.04 N HCl; it is not coagulated by heat. In a concentration of 2.5 mg. per cc. it gives only a slightly positive xanthoproteic test, and a pink biuret test, while pepsin gives a violet color. The Millon and Hopkins-Cole tests are negative. Rennin also differs from crystalline pepsin in that rennin diffuses through membranes if dialyzed long enough. Moreover, pepsin is, as found by Northrop (7), reversibly inactivated by alkali and is not very resistant to acids; whereas, the behavior of rennin is just the opposite in both respects. Like pepsin, rennin is also precipitated by saturated solutions of neutral salts as NaCl,  $\text{MgSO}_4$ , and  $(\text{NH}_4)_2\text{SO}_4$ . It is for this reason that pepsin-free rennin preparations were never obtained by the salting out methods. We found the isoelectric point of rennin in 50 per cent alcoholic solution to

be 5.4. This is far above that of crystalline pepsin, which is 2.75 as found by Northrop (6). Purified rennin is soluble in water at its isoelectric point while pepsin and impure rennin preparations are not. The most active preparation we could obtain had an activity of 1:4,550,000 when skim milk with  $\text{CaCl}_2$  was used as a substrate, but it lost about 50 per cent of its potency on drying. This is more than 100 times as active as Parke, Davis and Company's 1:30,000 rennin and about 2000 times as active as our original extract of pH 5.2. If the skim milk is made to contain 0.2 per cent lactic acid, the activity is about doubled, which means that 1 gm. of enzyme will clot 9 million gm. of milk. This rennin contains no pepsin as was shown by the fact that it did not produce an increase in formol titration and at most, only a negligible increase in N not precipitable by trichloroacetic acid, at pH 2.0 with coagulated egg white as substrate. This

TABLE I

*Composition of Most Active Rennin Preparation, Dried at 100° in Vacuo*

C	H	N, Dumas	P	Cl	S	Ash
61 33	7.02	14 40	0.00	0 00	1.19	0.40

would make our preparation the most active rennin yet reported. The elementary composition of our rennin preparation (Table I) and the above properties indicate that it is a thioprotease. It is not known whether the presence of proteoses in plant and animal tissues is due to the action of proteolytic enzymes on simple proteins or whether they are original constituents of the tissues. It should also be noted from the elementary analysis that rennin does not form a firm combination with Cl (HCl) and also that P is absent; both of these elements are constituents of the pepsin molecule (6).

*Determination of Rennet and Peptic Activity and the Preparation of Substrates*

For the determination of rennet activity the amount of enzyme which will clot 10 cc. of milk in 10 minutes at 40° was determined. For the preparation of the milk 500 cc. of distilled water are boiled

and 5 cc. of 8 per cent  $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$  added. The addition of  $\text{CaCl}_2$  within certain limits is favorable to rennet action (8). 50 cc. of this solution are added to 50 gm. of dry skim milk<sup>1</sup> and stirred with a rod for about 5 minutes. There results an evenly distributed pasty fluid. Now boiling water is added to the pasty mixture and if the milk is of good quality it will contain no lumps. After mixing and cooling the milk is ready for use. In our experiments fresh milk was made up every day. The enzyme solution for testing rennet activity was prepared by dissolving 100 mg. of the dry enzyme in 100 cc. of 0.04 N HCl and diluting 10 to 25 times with water according to its activity. To 10 cc. samples of milk various amounts of the enzyme solution are added; they are placed in a water bath at 40° and the time required for clotting observed.

It was stated by Fenger (5) that, if to cow's milk lactic acid is added to about 0.2 per cent, the clotting time can be shortened to about one-fifth. We found, too, that this is the case with fresh cow's milk, but when we used skim milk prepared as above we only noticed a decrease of half of the clotting time.

For the determination of peptic activity two methods were employed: (1) Northrop's (9) formol titration method was used with coagulated (boiled) egg albumin as a substrate; (2) the increase of N not precipitated by 10 per cent trichloroacetic acid was determined by the micro-Kjeldahl method. Here, too, coagulated egg albumin was used as a substrate. 1 cc. of the rennin solution containing 25 mg. of enzyme per cc. was added to 5 cc. of the suspension of coagulated egg albumin which contained 5 per cent dry weight of albumin, adjusted to pH 2.0, and incubated at 37° for 3 hours.

The following experiments, in which our highly purified rennin was used, corroborate earlier work on impure rennin as regards its behavior toward changes in pH.

#### *Effect of pH on Rennin Solutions*

50 mg. of rennin were dissolved in 0.4 N HCl, adjusted to pH 10 with NaOH at 22°, and 45 minutes later titrated to pH 5.5 with

<sup>1</sup> A standard dried skim milk preparation of the same type as used in their laboratory was kindly furnished us by Chr. Hansen's Laboratory, Inc. We are also indebted to them for the concentrated dry salted out rennin which they so kindly prepared for us.

HCl. It was allowed to stand at 22° for 18 and 24 hours and rennet activity determined. There was no rennet activity after 18 and 24 hours respectively.

50 mg. of rennin were dissolved in 0.04 N HCl and adjusted to pH 5.5 with NaOH at 22°. 18 and 24 hours later the rennet activity was determined. The rennet activity was practically unchanged.

These results indicate that rennin, unlike pepsin, is irreversibly and completely inactivated by alkali, but is fairly stable in dilute acid solutions.

#### DISCUSSION

In the attempts at purification of enzymes, two methods have been followed in the past: first, after certain preliminary procedures, crystallization has been sought, a method originally pursued by Pekelharing (1) in 1902. The first to succeed along these lines was Sumner (10) whose method for the isolation of crystalline urease is well known. Recently three more enzymes have been crystallized: pepsin by Northrop (6), trypsin by Northrop and Kunitz (11), and amylase by Caldwell, Booher, and Sherman (12). All of these were found to be proteins, and some corroboration has been given by Tauber (13), recently by Tauber and Kleiner (14), and more recently by Zakowski (15). Waldschmidt-Leitz, and Steigerwaldt (16) deny that enzymes are proteins, saying that the crystalline proteins are only carriers of the active enzymes. The controversy will probably continue until settled by some absolutely rigid method of separation.

The second method of purifying enzymes is the adsorption method of the Willstätter school. Inorganic substances are used as adsorbents and highly active preparations have been obtained. In the case of rennin, however, this procedure has not given satisfactory results.

As described above, our attempts to crystallize rennin yielded only some globular bodies, which could not be obtained in sufficient amount for quantitative determinations of any sort. When the new method was employed extremely active rennin was obtained. Thus far our most potent product had a rennet activity of 1:4,550,000 when  $\text{CaCl}_2$ -milk was used as a substrate. This substrate had a pH of 6.2, but if the acidity is increased by the

addition of lactic acid (Fenger (5)) to a pH of 5.4, the activity was about doubled. The importance of stating the pH of the substrate when discussing rennet activity is self-evident. This highly active rennin is thus 2000 times as active as our first extract and over 100 times as active as the usual commercial 1:30,000 preparations.

Our rennin, in contrast to that of Fenger and of Lüers and Bader, did not show any peptic activity. We found Northrop's crystalline pepsin<sup>2</sup> to have a rennet activity of 1:800,000 (CaCl<sub>2</sub>-milk as a substrate). This indicates that the peptic and rennet activity are not reversed in pepsin. Rennin has milk-clotting power but no peptic activity (*i.e.* proteolytic power at pH 2); whereas, pepsin has both peptic and rennet properties.

The two purified enzymes differ in various ways. Among them are the following: Rennin is irreversibly inactivated by alkali; it is very easily soluble in dilute acid; it is not coagulated by heat; it gives quite different protein color tests; and it is dialyzable. Moreover, its isoelectric point is 5.4 while that of pepsin is 2.75; and it is soluble in water at its isoelectric point, whereas pepsin is not. The various properties given and its chemical composition give evidence that rennin is a thioprotease, whereas pepsin is a simple protein. The elementary composition (Table I) shows that rennin, as obtained by our method, is free of Cl, which indicates that the latter is only in loose combination with the enzyme molecule in the form of HCl as a result of acid extraction and is separated in the course of preparation (dialysis, isoelectric fractional precipitation). Furthermore, it does not contain phosphorus. The absence of these two elements from the rennin molecule makes it distinctly differ from pepsin, since Northrop (6) found both Cl and P to be the constituents of the pepsin molecule.

It is of interest to note that Lüers and Bader's rennin having an activity close to ours had a nitrogen content of only 0.6 per cent as compared with the 14.4 per cent nitrogen in ours (Table I). Since Lüers and Bader's preparations still had considerable peptic activity which would account for some of the rennet action, it is

<sup>2</sup> A sample of crystalline pepsin, to which we refer whenever we mention "crystalline pepsin," was very kindly furnished by Dr. J. H. Northrop of the Rockefeller Institute.



evident that their rennin was not so highly purified as ours. It also seems possible that our preparation contains some impurity of high nitrogen content.

#### SUMMARY

An extremely active rennin preparation free of peptic activity has been obtained from an extract of the fourth stomach of the calf by fractional isoelectric precipitation. Its composition and characteristics show that rennin is a thioprotease. Numerous properties are described which prove that rennin and pepsin are distinct entities.

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## STUDIES ON RENNIN

### II. THE ISOLATION OF PRORENNIN\*

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#### INTRODUCTION

For many years it has been assumed that rennin in the mucosa of the fourth stomach of the calf is present in an inactive form and becomes active if in contact with HCl. No absolute proof for or against this theory has been furnished up to date, although Hedin (1) attempted to produce proofs against the existence of a zymogen by showing that blood serum has a retarding effect on rennin solutions, which disappears when HCl is added to the serum. Because of this, Hedin thought that "natural inhibiting substances" are responsible for the very slight activity of "neutral" extracts of the calf stomach and that the HCl makes these substances harmless. He thought that the zymogen is nothing but a mixture of active rennin with the inhibiting substances.

In 1922, Holwerda (2) found that neutral extracts of dried calf stomachs were inactive and could be activated after incubation for 3 days either at pH 4.7 at 25° or at pH 5.1 to 5.3 at 37°, and that at other acidities the activation is either very slow or absent. The experiments to be described will furnish distinct evidence, we believe, for the existence of a precursor of rennin.

#### EXPERIMENTAL

##### *Preparation of Neutral Calcium Carbonate Extract*

To 75 gm. of washed and minced mucosa of the fourth stomach of the calf 150 cc. of a 2 per cent suspension of calcium carbonate

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powder in distilled water are added, stirred with a rod for 8 minutes, and filtered through filter paper. The filtration is slow but a clear filtrate can be obtained on first filtration, the pH of which is 7.4.

*Experiment 1. Preformed Rennet Activity*—1 cc. of the original calcium carbonate extract clots 30 cc. of milk<sup>1</sup> in 10 minutes at 40°, without the addition of acid to the extract.

*Effect of pH on Calcium Carbonate Extract*

*Experiment 2*—50 cc. of the original extract of pH 7.4 were incubated 2 days at 37°; activity, none. After adjustment to pH 1 on the 3rd day the activity was 1:860.

*Experiment 3*—50 cc. of the original calcium carbonate extract were adjusted to pH 5.3 with 0.01 N HCl; the slight precipitate was not filtered off. Immediate activity, 1:333; activity after 1 day's incubation at 37°, 1:420.

*Experiment 4*—50 cc. of the original calcium carbonate extract were adjusted to pH 1 with 0.04 N HCl. Immediate activity, 1:2300; activity after 1 day at 37°, 1:1800.

*Experiment 5. Effect of Ethyl Alcohol upon the Calcium Carbonate Extract.*—To 50 cc. of the original calcium carbonate extract 50 cc. of 95 per cent ethyl alcohol were added; the insoluble, inactive precipitate and the supernatant fluid could not be activated at pH 1 to 2 even after 3 days at 37°.<sup>2</sup>

*Experiment 6. (a) Preparation of Zymogen, Prorennin, Free of Rennet Activity*—The calcium carbonate extract was carefully adjusted to pH 9 to 10 at 22° and left at this temperature for 40 minutes to destroy any active rennin and pepsin. To the inactive solution, solid MgSO<sub>4</sub> was added to saturation, after which a precipitate formed, which, on centrifuging, separated as a cake on the top of the solution. This cake was dissolved at 22° in the same volume of 0.25 saturated MgSO<sub>4</sub> as the original solution, filtered to free it from insoluble matter, and reprecipitated with solid MgSO<sub>4</sub> as before. The precipitated prorennin, after being centrifuged, was dried *in vacuo* over sulfuric acid at room temperature.

*(b) Showing That Prorennin Is Inactive and Can Be Activated*

<sup>1</sup> The same calcium chloride milk as described in the previous paper was used.

<sup>2</sup> In these experiments toluene was used as an antiseptic.

CORRECTION

756, Vol. 96, No. 3, June, 1932, Experiment 4, line  
0.04 N HCl.

by *HCl*—50 mg. of the dry substance were dissolved in 10 cc. of distilled water by keeping them for 40 minutes in a water bath at 42°. After this, the solution was filtered and a water-clear filtrate obtained.

To 1 cc. of the clear filtrate 1 cc. of 0.04 *N HCl* was added, mixed, and 1 cc. diluted with 4 cc. of water. 1 cc. of this diluted solution clotted 10 cc. of milk in 11 minutes at 40°.

To 1 cc. of the clear filtrate 5 cc. of distilled water (instead of *HCl*) were added. 1 cc. of this solution did not clot 10 cc. of milk at 40° even after 3 hours.

#### DISCUSSION

In Experiment 1 it has been shown that aqueous  $\text{CaCO}_3$  extract of the mucosa of the fourth stomach, pH 7.4, has only slight rennet activity if immediately tested. This may be due to rennin already present as such in calf mucosa, activated by the *HCl* of the stomach, or more probably to traces of prorennin secreted by some virile cells and activated by *HCl* similarly secreted. This active rennin of the calcium carbonate extract, since it is not stable at pH more than 6, becomes inactive when left over 2 to 3 days at 37°, but, if adjusted to pH 1, the inactive precursor becomes active. If the original extract is brought to pH 5.3, activation takes place; but as seen in Experiment 3, this is very slow. Experiment 4 demonstrates that the prorennin can fully and immediately be activated if adjusted to pH 1 with *HCl*. Further incubation does not increase the activity; on the contrary a decrease can be noted. Therefore, we cannot confirm Holward's statement that only at 4.7 and 5.1 to 5.3 respectively does activation take place and then only at a very slow rate (3 days). In our Experiment 3 we found that at pH 5.3 activation is slow, but immediate activation may be attained at pH 1. In Experiment 5 it is shown that if 95 per cent alcohol is added to a concentration of about 50 per cent, complete destruction of the zymogen takes place; this makes prorennin distinctly differ from rennin (see preceding paper). Possibly the molecule of prorennin is more easily modified by ethyl alcohol than that of rennin. By the precipitation with alcohol the prorennin becomes insoluble and permanently inactive. It cannot be activated even after days in contact with *HCl*, but the precursor can be obtained in a water-soluble

form, free from rennet activity by salting out the calcium carbonate extract with  $\text{MgSO}_4$  (Experiment 5). Our experiments exclude the possibility of the existence of "natural inhibiting substances" of Hedin's. There is no need to search for special inhibiting substances as was done by Hedin in his studies of serum proteins, since the inhibition produced by him was due only to the protective colloidal action of the serum proteins which delayed the precipitation of the calcium paracaseinate formed. The fact is that the prorennin, as shown in Experiment 5, is present in the mucosa in an inactive form and is activated by  $\text{HCl}$ .

#### SUMMARY

1. Experimental evidence has been given, which proves the existence of a precursor of rennin.
2. The precursor has been isolated in a water-soluble dry form, free of rennet activity.

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## LIPID\* EXCRETION

### VIII. THE LIPID CONTENT OF THE INTESTINAL MUCOSA†

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The existence of a fecal lipid excretion, independent of the diet, has been demonstrated in previous work. Normal and bile fistula dogs were maintained on a lipid-free diet for intervals of time varying from 1 to 5 weeks, during which the feces were marked off, collected, and analyzed for their lipid content in periods of 1 week. Altogether 83 such experiments were carried out with normal and forty-three with bile fistula dogs. Most of the results, expressed as total petroleum ether-soluble substances excreted per dog per week, have been presented in previous papers of this series (1, 2). The data have been recalculated in terms of excretion of unsaponifiable plus fatty acid fractions<sup>1</sup> per kilo of dog weight per week for comparison with the results obtained in this and the following investigations. The average excretion thus expressed was  $219 \pm 9.5$  mg. for normal dogs and  $615 \pm 25.2$  mg. for bile fistula dogs.

A possible source of the excretion is desquamation of intestinal

\* The word "lipid" is used as a general term including fats and associated substances. In this and other papers of this series it has been used to denote substances soluble in petroleum ether.

† A preliminary report of this investigation was made before the American Society of Biological Chemists at Montreal, April, 1931.

This investigation was supported in part by funds supplied by the Chemical Foundation.

<sup>1</sup> In most of the experiments the sum of the unsaponifiable and fatty acid fractions was from 5 to 10 per cent lower than the petroleum ether-soluble fraction before separation.

epithelium containing fatty substances. There are few data in the literature on the lipid content of intestinal epithelium or mucosa. Ewald (3) determined the total ether-soluble material in three samples of intestinal mucosa from two dogs. He found an average of about 6.0 per cent, based on the dry weight of the mucosa. Moore (4) studied the ether-soluble substances in epithelium scraped from the small intestine in two dogs 7 hours after a large dose of olive oil. He found an average of about 4.5 per cent ether-soluble substances in moist epithelium. Noll (5) determined the petroleum ether-soluble substances in the epithelium from three fasting rabbits, and obtained an average of about 5.0 per cent on the basis of dry epithelium. Mueller (6) determined free and combined cholesterol in the epithelium from three postabsorptive dogs, but he does not give the weights of epithelium. Sinclair (7) studied the phospholipid content of the mucosa in a number of cats and obtained values ranging from 5.05 to 12.45 per cent on the basis of dry weight.

The purpose of the present investigation was to obtain further information concerning the lipid content of the intestinal mucosa and to employ the results in estimating the maximum possible rôle which desquamation may play in lipid excretion.

#### EXPERIMENTAL

The entire intestine was removed as soon as possible after death from dogs which had not eaten for at least 24 hours. The external muscular layers were stripped from the mucosa by a slight modification of the method employed by Sinclair (7). The muscle was freed from the mucosa at the one end by a procedure similar to Sinclair's, and then the mucosa was drawn out between the thumb and the forefinger, by means of which the muscle was turned back over the intestine. With a little practice the mucosa could be freed almost completely from muscle in 10 to 15 minutes. A few muscle fibers remained adherent to the mucosa, but this fact, if anything, tended to strengthen rather than weaken the final interpretations. The mucosa from the small intestine was divided into two equal portions according to length, and these, as well as the mucosa from the colon, were analyzed separately. The mucosa was first opened longitudinally, washed thoroughly in running water, cut up into small pieces, and placed in 95 per cent ethyl



alcohol. Each sample was then extracted in a continuous hot extractor (2) for from 4 to 6 hours, three to four fresh portions of alcohol being used, after which it was extracted three or four times with cold ether, dried, and weighed. The combined extracts were evaporated to dryness, taken up in ether, and the ether-soluble material was weighed, saponified with 20 per cent alcoholic KOH, and separated into unsaponifiable and fatty acid fractions. The residue, extracted by alcohol but insoluble in ether, was also weighed. This weight, together with the amounts of ether-soluble material and extracted mucosa, gave the dry mucosa weight.

Cholesterol was determined in the unsaponifiable fractions by the Yasuda<sup>2</sup> (8) modification of the Okey<sup>2</sup> (9) method, except that an electric heater instead of a steam jacket was used to keep the alcohol hot in the filtration funnel. All determinations were made in duplicate. A 1.0 mg. sample of cholesterol was analyzed along with every four or eight determinations. Recoveries varied from 96 to 102 per cent.

#### DISCUSSION

The dogs used were classified into three groups, as shown in Table I. The six dogs in the first group were taken from stock and, as far as is known, were entirely normal. The five dogs in the second group could not be classed as strictly normal, though their abnormalities probably had no effect on the intestinal tract. The six dogs in the third group had had ileostomies performed and were definitely abnormal.<sup>3</sup>

Table I shows the amounts of lipids<sup>4</sup> present in the three portions of mucosa studied. No significant difference is apparent among the different groups of dogs or between the lipid content of the upper and lower portions of the small intestine, but in sixteen of the seventeen experiments the colon mucosa contained a smaller proportion of fatty substances than the small intestine mucosa. The difference in averages is 1.5 per cent of the dry mucosa weight.

<sup>2</sup> The author is indebted to Dr. Okey and Dr. Yasuda for furnishing details of their methods before publication.

<sup>3</sup> See the following paper for a discussion of their condition.

<sup>4</sup> The values for lipids represent the sum of the unsaponifiable and fatty acid fractions.

TABLE I  
*Lipid Distribution in Mucosa Segments*

Dog No.	Dog weight	Weight of dry mucosa			Weight of lipids			Lipids in dry mucosa				
		Small intestine		Colon	Small intestine		Colon	Small intestine			Colon	
		Upper half	Lower half		Upper half	Lower half		Colon	Upper half	Lower half		Total
Group I, normal dogs												
	kg.	gm.	gm.	gm.	gm.	gm.	gm.	per cent	per cent	per cent	per cent	
1	7.0*	9.4	6.6	2.4	0.689	0.592	0.128	7.3	9.0	8.0	5.3	
2	4.8	12.0	7.0	3.1	1.048	0.566	0.233	8.7	8.1	8.5	7.5	
3	6.2	8.9	6.5	2.7	0.615	0.429	0.208	6.9	6.6	6.8	7.7	
4	12.8	14.2	14.1	7.3	1.261	0.934	0.296	8.9	6.6	7.8	4.1	
5	5.0	9.4	6.8	2.7	0.831	0.550	0.155	8.8	8.1	8.5	5.7	
6	7.2	18.1	11.1	4.5	1.352	0.817	0.312	7.5	7.4	7.4	6.9	
Average .....								8.0	7.6	7.8	6.2	
Group II, dogs with abnormalities but with normally functioning intestines												
7†	23.0*	26.0	13.8	5.0	1.657	0.850	0.310	6.4	6.2	6.3	6.2	
8‡	6.4	12.3	8.8	3.0	0.880	0.680	0.193	7.2	7.7	7.4	6.4	
9‡	8.7	15.1	12.2	4.8	1.330	0.794	0.368	8.8	6.5	7.8	7.7	
147§	10.7	16.3	12.2	3.7	1.448	1.205	0.258	8.9	9.9	9.3	7.0	
203§	8.8	13.8	9.0	3.2	1.119	0.713	0.210	8.1	7.9	8.0	6.6	
Average .....								7.9	7.6	7.8	6.8	
Group III, dogs with ileostomies												
238	14.5	18.6	14.0	3.8	1.623	1.290	0.233	8.7	9.2	8.9	6.1	
239	4.3	4.7	3.2	1.2	0.299	0.244	0.076	6.4	7.6	6.9	6.3	
251	6.0	7.2	3.9	1.8	0.635	0.378	0.134	8.8	9.7	9.1	7.4	
623	12.7	20.4	15.7	4.9	1.319	0.945	0.250	6.5	6.0	6.3	5.1	
647¶	7.0*	9.2	6.9	2.7	0.578	0.467	0.120	6.3	6.8	6.5	4.4	
720	8.3	17.5	10.5	2.9	1.431	0.893	0.202	8.2	8.5	8.3	7.0	
Average .....								7.5	7.9	7.7	6.1	
" of all experiments .....								7.8	7.7	7.8	6.3	
										±0.16**	±0.18**	

\* Approximation.

† Dog 7 had been subjected to an operation involving the spleen; it had not eaten for 4 to 5 days before sacrifice.

‡ Slight distemper.

TABLE I—*Concluded*

§ Cecostomies had been performed but dogs were essentially normal. See following paper.

|| Did not retain food and was sacrificed 4 days after operation.

¶ Did not retain food and died 5 days after operation.

\*\* Probable error of mean.

This difference was shown to be significant by calculating that its standard error is 0.34 from the formula

$$\frac{\sigma_{SI}}{N}$$

where  $\epsilon$  is the standard error,  $\sigma_{SI}$  is the standard deviation of the small intestine percentages,  $\sigma_C$  is the standard deviation of the colon percentages, and  $N$  is the number of observations (10).

Table II shows the distribution of unsaponifiable material in the mucosa. The average percentages in the dry mucosa are slightly higher in the case of the lower half of the small intestine and the colon than in the upper half of the small intestine. The average percentage of unsaponifiable material in lipids is also somewhat greater in the lower than it is in the upper half of the small intestine. Statistical analysis shows, however, that these differences are not significant.

Since the colon contained a smaller amount of lipids than the small intestine and at least as much unsaponifiable material, it follows that the proportionate amount of unsaponifiable material must be higher in the lipids of the colon than in the lipids of the small intestine. This is true in fifteen of the individual experiments. The difference in averages is 5.5 per cent and the standard error of this difference is 1.8 per cent, a result which is only just within the limit considered significant by Yule. In this treatment variations between different dogs in the percentages under consideration increase the standard error of the difference in averages. When the *individual* differences are treated according to "Student's" method as given by Fisher (11), it turns out that the odds are better than 100 to 1 that the mean difference is significant.

The foregoing considerations lead to the conclusion that the

small intestine mucosa contains a higher proportion of fatty acids<sup>5</sup> than the colon. This may be related to the greater metabolic

TABLE II  
*Distribution of Unsaponifiable Material in Mucosa Segments*

	Dog No.	Unsaponifiable material in dry mucosa			Unsaponifiable material in lipids			
		Small intestine		Colon	Small intestine			Colon
		Upper half	Lower half		Upper half	Lower half	Total	
		per cent	per cent		per cent	per cent	per cent	
Group I	1	1.91	1.92	1.75	26.0	21.4	23.7	32.8
	2	1.33	1.43	2.06	15.2	17.8	16.1	27.5
	3	1.36	1.36	1.96	19.7	20.5	20.0	25.5
	4	1.62	1.27	0.88	18.3	19.2	18.6	21.6
	5	1.36	1.35	1.15	15.4	16.7	15.9	20.0
	6	1.18	1.52	1.27	15.8	20.7	17.6	18.3
Average.....		1.46	1.47	1.51	18.4	19.4	18.6	24.3
Group II	7	1.07	1.22	1.32	16.7	19.8	17.8	21.3
	8	1.36	1.69	1.43	19.0	21.9	20.3	22.3
	9	1.41	1.40	1.33	16.0	21.4	18.0	17.4
	147	1.46	1.33	1.84	16.4	13.4	15.1	26.3
	203	1.36	1.57	1.66	16.8	20.0	18.0	25.2
Average.....		1.33	1.44	1.52	17.0	19.3	17.8	22.5
Group III	238	1.35	1.58	1.92	15.4	17.1	16.2	31.3
	239	1.72	1.84	2.66	27.0	24.2	25.8	42.1
	251	1.97	1.87	1.89	22.4	19.3	21.2	25.4
	623	1.12	1.21	1.20	17.4	20.1	18.5	23.6
	647	1.03	1.39	0.56	16.4	20.5	18.3	12.5
Average.....	720	2.12	2.74	2.07	25.8	32.2	28.2	29.8
	of all experiments	1.55	1.77	1.71	20.7	22.2	21.4	27.4
		1.45	1.57	1.58	18.8	20.4	19.4	24.9
		±0.052	±0.039	±0.084	±0.56	±0.63	±0.59	±1.11

activity of the former, since the fatty acids were probably present

<sup>5</sup> The proportion of fatty acids, of course, varied inversely with the unsaponifiable fraction since the lipid values represented the sum of the two.

largely in phospholipids, which appear, from the work of Bloor (12), to vary more or less in proportion to metabolism.

Table II also shows that the average unsaponifiable content of all three mucosa segments is higher, both in percentage of dry

TABLE III  
*Lipids in Entire Mucosa*

	Dog No.	Lipids in entire dry mucosa		Unsaponifiable material	
		Per cent	Mg. per kilo dog weight	Mg. per kilo dog weight	Per cent in lipids
Group I	1	7.7	201*	49.7*	24.7
	2	8.4	384	67.5	17.6
	3	6.9	202	42.2	20.9
	4	7.0	195	37.0	19.0
	5	8.1	307	50.2	16.3
	6	7.4	345	61.0	17.7
Average.....		7.6	287	51.6	19.4
Group II	7	6.3	122*	22.2*	18.2
	8	7.3	274	56.0	20.4
	9	7.8	286	51.4	18.0
	147	9.0	272	43.7	16.1
	203	7.9	232	43.7	18.8
Average.....		7.7	266	48.7	18.3
Group III	238	8.7	217	37.6	17.3
	239	6.8	144	40.0	27.8
	251	8.9	191	41.5	21.7
	623	6.1	198	37.6	19.0
	647	6.2	166*	29.4*	17.7
	720	8.2	305	86.5	28.4
Average.....		7.5	211	48.6	22.0
" of all experiments.....		7.6	254	49.7	20.0

\* Approximate; not included in averages.

mucosa and of lipids, in Group III than in the other groups. This finding, if significant, would be consistent with the decreased metabolism of the mucosa which must have been present in the dogs of this group. It is interesting that the values found with

Dogs 623 and 647 are lower for the most part than the corresponding averages of Groups I and II. Dogs 623 and 647 died 4 and 5 days (respectively) after operation (see Table I). The other four dogs in Group III lived from 11 to 18 days after operation and were used in experiments described in the following paper. The averages from these four dogs are considerably higher than the corresponding averages of Groups I and II.

In order to conserve space the results of cholesterol determinations in the unsaponifiable fractions are not given in detail. No significant differences were found between the three groups of dogs or the three portions of the intestine studied. Low results were obtained more often in Group III than in the other groups, but no significance is attached to this finding. The average of all the data is  $50.8 \pm 1.5$  per cent.

The results for the lipids of the entire mucosa (sum of the three portions analyzed) are presented in Table III. The percentage of lipids in the dry mucosa is quite uniform throughout. No differences are apparent in the three groups of dogs.

A considerable variation is noted in the amounts of lipids per kilo of dog weight in the different animals. Since the percentages of lipids in the mucosa were relatively uniform, it follows that the variation is due primarily to differences in the amount of mucosa per kilo. The average amounts of lipids per kilo are in very close proportion to the average amounts of mucosa, which are 3.75 gm. for Group I, 3.36 gm. for Group II, and 2.75 gm. for Group III.

The primary purpose of this investigation was to obtain data from which the possible rôle of desquamation of intestinal epithelium in lipid excretion might be estimated. From the average lipid excretion per kilo (see p. 759) and the average lipid content of the mucosa per kilo (Table III) it may be calculated that 12.4 per cent of the entire mucosa would have to desquamate each day to account for the excretion. It may be objected that an estimate based on average values is unjustified since there are such large variations in individual experiments, particularly in lipid excretion. In the extreme and unlikely case that the dog with the lowest excretion found also had the highest mucosa lipid content, a desquamation of 2.4 per cent of the mucosa per day would still be necessary to account for the excretion.

It happens that the mucosa lipids contained a smaller percentage of unsaponifiable material than the excreted lipids (20.0 as

compared with 37.8 per cent). When calculated as above, on the basis of average unsaponifiable values, it is found that a desquamation of 24.0 per cent of the entire mucosa per day would be necessary to account for the unsaponifiable excretion.

Similar calculations from the results obtained with bile fistula dogs lead to the conclusion that a desquamation of 34.6 per cent of the entire mucosa per day would be necessary to account for the average lipid excretion, while 59.6 per cent would be necessary to account for the average unsaponifiable excretion.

It is probable that desquamation is confined to the superficial epithelial layers. Since these contain only a *part* of the total mucosa lipids, it follows that a larger—probably a much larger—proportion of the *epithelium* than is indicated by the above estimates would have to desquamate per day to furnish the lipids found in the feces. No information has been found concerning the probable amount of desquamation, but in a normal animal it seems most unlikely that it can be sufficient to provide a significant source of the endogenous lipid excretion.

While this work was in progress Gardner and Gainsborough (13) pointed out that desquamation could hardly be a factor in lipid excretion, since a desquamation of 100 gm. of material per day by a 10 kilo, bile fistula dog would be necessary to account for excretions reported previously by the author. The authors do not state their source of information concerning the mucosa cholesterol content, on which their calculation must have been based. The results of this investigation are in entire agreement with the estimate of Gardner and Gainsborough.

#### SUMMARY AND CONCLUSIONS

1. From a comparison of the lipid content of the mucosa and the amounts of lipids excreted by dogs on a lipid-free diet it is concluded that desquamation of intestinal epithelium in all probability is not an important source of the endogenous lipid excretion.

2. The colon mucosa contained a smaller amount of fatty acids than the small intestine mucosa. A tendency toward a smaller content of fatty acids was found in the mucosa of dogs which had ileostomies than in the mucosa of normal dogs. It is suggested that these findings may be related to the metabolic activity of the mucosa.

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## LIPID EXCRETION

### IX. THE SECRETION OF LIPIDS INTO THE INTESTINE\*

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In a previous paper (1) the bile, bacterial synthesis, desquamation of intestinal epithelium, and secretion into the intestine were suggested as possible sources of the endogenous lipid excretion which had been found to occur in dogs. Evidence (2) has been presented to show that in all probability bile is not the source of the excretion. Studies of bacterial synthesis (3, 4) were not conclusive, but it was indicated that not over 40 per cent and probably a much smaller part of the excretion originated in bacteria. This point will be further discussed below. In the preceding paper it is shown that desquamation of epithelium in all probability does not play an important rôle in the excretory process. Only secretion of lipids into the intestine remains to be considered.

Recent work by German investigators indicates that the cholesterol portion of the endogenous excretion represents a secretion into the large bowel. Beumer and Hepner (5) found that the cholesterol in the intestinal contents of a normal dog during absorption is much higher, based on percentage of dried weight, in the colon than in the ileum. They also studied a bile fistula dog after a lipid-free meal and found an even more marked difference; 0.21 per cent cholesterol in dried ileum contents and 1.25 per cent in

\* A preliminary report of this investigation was made before the American Society of Biological Chemists at Chicago, March, 1930.

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the contents of the colon. They interpreted their results to indicate a secretion of cholesterol into the large bowel. Bürger and Oeter (6) found a definitely greater cholesterol content of the intestinal wall of the sigmoid than of various sections of the small intestine in cadavers. They interpreted this finding to indicate passage of cholesterol across the wall of the large intestine and they thought it must be going into the lumen and not out, because no coprosterol was found. In the preceding investigation no difference could be made out in the cholesterol content of the colon and the small intestine mucosa, but it must be remembered that these results were obtained with dogs, while Bürger and Oeter worked with cadavers. Angevine (7) recently confirmed older work showing a very appreciable secretion of lipids from isolated loops of intestine.

The purpose of the present investigation was to study the secretion of lipids into the intestine by ascertaining whether the endogenous excretion originates in the small or large intestine; or, if in both, as was actually the case, to determine the relative amounts coming from the two portions. The general plan of the work was as follows: Fistulas of the small intestine were made in dogs just above the ileocecal valve, the colon being tied off and left in place. In a few cases cecostomies were performed. The dogs were maintained on a lipid-free diet and the excretion from the fistulas was collected in pads of gauze from which the lipids were extracted and analyzed. The colon was flushed out at the beginning of the experiment by enemas and the lipids which had collected were determined in the contents obtained at autopsy.

#### EXPERIMENTAL

*Operative Technique*<sup>1</sup>—Ileostomies were performed by sectioning the intestine between ligatures at a point about 10 cm. from the cecum. The distal end was inverted and tied firmly. The proximal end was passed into a test-tube which was then drawn

<sup>1</sup> The authors are indebted to the Departments of Surgery of The University of Rochester School of Medicine and Dentistry and the College of Physicians and Surgeons of Columbia University, and to the New York State Psychiatric Institute for operating facilities. They are also indebted to Dr. William J. Heeks for performing the operations on Dogs 607 and 720.

out through a stab wound in the right flank. Thus any contact between the cut end of the intestine and the tissues of the abdominal wall was avoided. The protruding intestine was anchored firmly to the fascia and skin by a number of sutures. Fistulas resulted which in each case remained open until the termination of the experiment.

*Diet*—In most of the experiments the dogs were placed on a diet of lean Hamburg steak on the day following the operation and were kept on this diet until they were eating well (usually 3 or 4 days), when they were given the same lipid-free diet which has been used in previous work, with the omission of bone ash and meat extract. The food was suspended in warm water and fed by stomach tube throughout. In a few cases (Experiments 14 to 19) the experimental diet was started on the day following the operation.

*Condition of Dogs*—Most of the dogs remained in fairly good condition during the 1st experimental week, but sometime during the 2nd week they commenced to vomit, became sluggish, and died or were sacrificed 3 to 5 days after the inception of vomiting. In some cases there was also secretion from the nose, suggestive of distemper. Determinations<sup>2</sup> of a number of constituents of the blood were carried out frequently during the course of the experiments on Dogs 607, 307, and 720. Space does not permit a detailed exposition of the results. The principal finding was a large drop in the CO<sub>2</sub>-combining power coincident with the development of pathological symptoms. Dog 720 was treated, beginning the 6th day after operation, with clyses of 3 per cent glucose in normal saline and sodium bicarbonate throughout the remainder of the experiment. Under this treatment the CO<sub>2</sub>-combining power and the chlorides remained within normal limits until just before death, when the former showed a tendency to drop and the latter a tendency to rise. It is probable, from the foregoing observations, that the primary disturbance in these animals was an acidosis resulting from desiccation and loss of base.

Schönheimer and von Behring have published (8) experiments on dogs with ileostomies which were practically identical with ours. Their animals survived for as long as 3 months. The much

<sup>2</sup> The authors are indebted to Dr. Ruth Sullivan of the Department of Chemistry of the Babies Hospital for these analyses.

TABLE I  
*Lipids from Fistulas of Small Intestine*

Experiment No.	Dog No.	Dog weight*	Duration of experiment†	Petroleum ether-soluble fraction‡	Lipids		Unaponifiable fraction		
					Unaponifiable + fatty acid fractions	Per kilo dog weight per wk.	Weight	In lipids	Sterols in
					gm.	mg.			
1§	215	4.7	7	2.546 (0.103)	1.949	415	0.499	25.6	33.3
2			7	4.260 (1.209)	3.587	764	0.928	25.9	42.1
3§	216	12.3	7	0.612 (0.743)	0.449	37	0.144	32.0	
4			7	8.670 (3.924)	7.125	579	1.357	19.0	29.4
5			6	2.560 (None)	2.438	198	2.400	98.5	39.1
6	218	5.5	7	7.107 (3.281)	5.708	1037	1.795	31.5	18.4
7			4	3.762 (1.062)	3.298	600	1.209	36.6	20.2
8	229	11.0	7	10.982 (0.145)	6.568	597	2.020	27.3	34.5
9			6	10.120 (0.048)	6.905	628	2.072	30.0	55.4
10	235	4.7	7	12.633 (0.024)	7.820	1664	1.967	25.1	30.2
11			5	5.475 (0.035)	3.540	754	1.186	33.5	36.7
12	238	14.5	7	10.296 (0.073)	7.296	503	1.824	25.0	54.1
13	239	4.3	7	7.369 (0.007)	5.139	1194	1.564	30.4	38.7
14	607	20.0	7	19.086	16.640	832	4.100	24.6	
15	720	8.3	7	15.565	10.956	1320	2.045	18.7	60.9
16			5	8.850	7.009	845	2.156	30.8	58.9
17	307	11.8	7	15.771	11.309	960	1.161	10.3	
18			6	8.212	7.148	606	0.608	11.8	
19	405	6.1	4	10.825	9.039	1481	3.510	38.8	82.2
Average...								26.5	42.3

\* The dogs lost a good deal of weight during the experiments but the original weight was used in calculating the excretion per kilo.

† When the experiment was of less than 7 days duration, the results were calculated on the basis of a full week.

‡ Numbers in parentheses are ether-soluble, petroleum ether-insoluble fractions.

§ Extraction incomplete (see text).

|| Not included in the average.

*Dog #15*—Distemper signs shown during most of 2nd week. Vomiting on the 2nd and last 3 days of the 2nd week. Died just after the last collection.

*Dog #16*—Large, active dog. No abnormal signs until vomiting commenced on the 4th day of the 3rd week. Died on the morning of the 7th day.

TABLE I—*Concluded*

*Dog 218*—Vomiting commenced on the 2nd day of the 2nd week and the dog died on the 5th day.

*Dog 229*—Some nasal secretion noted on the 2nd day of the 2nd week. Slight vomiting on the 4th day increasing on the 5th and 6th. Sacrificed on the 7th day.

*Dog 235*—Vomiting on the 2nd day of the 1st week, continuing throughout week. None whatever on the first 5 days of the 2nd week. Despite retention of food the dog was very sluggish and became almost comatose on the 5th day, dying on the 6th.

*Dog 238*—No abnormal signs except for the presence of a number of worms in the intestine found at autopsy. Sacrificed at the end of the 1st week.

*Dog 239*—Vomiting commenced on the 2nd day of the 1st week and there was some vomiting every day thereafter until the dog was sacrificed at the end of the 1st week.

*Dog 607*—No vomiting or other abnormal signs. A good deal of excretion was lost in the experiment with this large dog through failure of the gauze to hold it.

*Dog 720*—Vomiting on last 3 days. Special treatment (see text).

*Dog 307*—Vomited on 2nd and last 3 days of 2nd week.

*Dog 405*—Vomited all 4 days.

shorter survivals of our dogs are probably explained in part by the fact that the gauze and canvas jacket used in collection (see next section) prevented the animals from reingesting the secretion, while in the description of their experiments nothing is said about any attempt to protect the fistulas. Without exception our dogs showed an intense desire to lick the fistulas and surrounding area when for any reason the dressing was removed. Another factor which probably affected the time of survival was the use by Schönheimer and von Behring of a more adequate diet of meat, eggs, and fish. Even so, their dogs lost a good deal of weight and, as they say, were kept alive only by very good care.

Heupke (9) performed a similar operation on a single dog, which lived 12 weeks. No attempt to prevent reingestion during this experiment is mentioned.

Brief statements concerning the condition of the individual dogs are attached to Table I.

*Collection of Excretion*—Collection was started on the 2nd day of the lipid-free diet. Much time was wasted in an attempt to collect the excretion by means of cannulas of different sorts, particularly the one used by Angevine previously (7). We were

unable to hold the cannula in place, as was done with the isolated loop fistulas, partly because of the lack of hair and partly because of the condition of the skin caused by the digestive action of the excretion. The problem was eventually solved by the use of a large pad of gauze held in place over the fistula by a binder of rubberized cloth which prevented leakage. The cloth binder was in turn completely covered and held in place by a heavy canvas jacket. The gauze was extracted thoroughly with hot alcohol for several hours in a continuous extractor and then washed several times with ether before use. Two gauze pads were used for each dog, one replacing the other each day. The one containing the excretion was placed in a continuous extractor (1) and extracted with hot alcohol for 4 to 5 hours or until the alcohol coming through the gauze was quite clear.

*Analytical Procedure*—The alcohol extracts were combined in weekly (or less as indicated in Table I) portions and the lipid content was determined. In Experiments 1 to 7 the method of complete saponification, which has been used in work with feces, was employed. The entire mixture was concentrated on the steam bath with the aid of an air jet. (Concentration *in vacuo* was attempted in Experiments 1 and 3 and was found to be very difficult due to frothing.) Sufficient strong aqueous NaOH was added to make the final concentration about 30 per cent and the mixture was digested at least 12 hours with frequent shaking. The digest was acidified with 5 N HCl (except in Experiments 1 and 3 where H<sub>2</sub>SO<sub>4</sub> was used), and distilled almost to dryness *in vacuo*. A thick, tarry paste was obtained, very difficult to extract with ether. It was particularly bad in Experiments 1 and 3, due perhaps to the use of H<sub>2</sub>SO<sub>4</sub>, and the extraction in these experiments was certainly far from complete. Up to forty washings, with thorough mixing each time, were found necessary to remove all the lipids in most of the experiments. The ether extract was washed with water, distilled to dryness on the steam bath, and the residue was extracted with petroleum ether. The petroleum ether extract was separated into unsaponifiable and fatty acid portions by the usual technique.

Because of the difficulty experienced with tar formation and frothing in distillation, the procedure was modified in Experiments 8 to 26 as follows: The combined extracts were taken to dryness

on the steam bath with the aid of an air jet and the residue was extracted several times with boiling alcohol and (afterwards) with ether. The combined extracts were filtered, distilled *in vacuo* until the alcohol was mostly removed, and then allowed to stand overnight in the presence of a fairly large amount of ether. The ether was decanted and the thick, rather tarry residue was washed several times with ether. The combined ether extracts and washings were taken to dryness on the steam bath, the residue was extracted with petroleum ether, and the petroleum ether extract was separated into neutral and free fatty acid portions. The neutral fraction was saponified with 20 per cent alcoholic KOH for 2 or 3 hours and then separated into unsaponifiable and fatty acid portions.

Digitonin-precipitable sterols were determined by the Yasuda modification of the Okey method as in the preceding investigation.

Usually in extracting lipids from any biological source there is a fraction obtained which is soluble in ether but insoluble in petroleum ether. Such a fraction has been noted in previous work with feces and has been measured in some cases. It has usually been found to be negligible in amount when compared with the petroleum ether-soluble portion. In work with isolated loops (7) this fraction was found to be equal in amount to the petroleum ether-soluble portion. In the present investigation this fraction was measured by extracting with dry ether the residue from the petroleum ether extract of the total ether-soluble substances, filtering if necessary, taking to dryness on the steam bath, and weighing.

#### DISCUSSION

The data obtained from ileostomy dogs are presented in Table I. It will be noted that in Experiments 1 to 7 where the saponification method was employed there was a considerable loss, ranging around 20 per cent, during the separation of the petroleum ether-soluble fraction into unsaponifiable and fatty acid portions. In previous work with feces the loss during the same separation was about 5 to 10 per cent in most experiments. In Experiments 8 to 13 where a different method was used, a much greater difference is evident between the petroleum ether-soluble fraction and the sum of the unsaponifiable and fatty acid portions.

This larger difference seems to be related to the ether-soluble, petroleum ether-insoluble portion (shown in parentheses), which is quite large by the first method but negligible by the second. Apparently this fraction remains petroleum ether-soluble in part at least until after saponification. As obtained in Experiments 1 to 7 it was very highly pigmented and tar-like in appearance. It was found to contain 12.7 per cent of alkali-insoluble material. How much of this fraction is lipid in nature is not known. It will be disregarded in the following discussion and attention will be focused on the lipid fractions found by adding the unsaponifiable and fatty acid portions. It should be emphasized that these are *minimum* values and represent somewhat more highly purified fractions than the total lipid values (petroleum ether-soluble substances) given in previous work with feces. The results obtained in previous work have been recalculated on the same basis (see p. 759, preceding paper).

It is evident that there was a large excretion of lipids from fistulas of the ileum in every experiment, regardless of the condition of the animal. Except in Experiments 1 and 3 where extraction was almost certainly incomplete, and Experiment 5 which presented unusual aspects and will be discussed below, the lowest excretion obtained in terms of dog weight was larger than the largest fecal excretion obtained in any of the 83 experiments carried out on normal dogs. In most cases the excretion was considerably smaller in the 2nd than in the 1st week. This is, in all probability, related to the fact that all of the dogs except Dog 216 vomited and were in generally poor condition during the 2nd week. It would be expected that the excretion would be smaller, since the intestine was less active in handling food and probably had a diminished secretory activity. It has been found (1) that fasting dogs excrete less lipids than dogs receiving a lipid-free diet. The fact that relatively large excretions were found, despite inanition, emphasizes the conclusion that there is a large secretion of lipids into the small intestine.

The average unsaponifiable content of the lipids is somewhat smaller than that found in the excretion of normal dogs. The average of all experiments except Experiment 5 is 26.5 per cent, while the average of all experiments with normal dogs, calculated on the same basis, was 37.8 per cent. For the most part individual



deviations from the average are not large, but the excretion from Dog 307 contained a relatively small amount of unsaponifiable material. On the other hand during the 3rd week on the diet (Experiment 5) Dog 216 excreted lipids which were composed almost entirely of unsaponifiable material. We have no explanation for this unusual result.

The finding that much larger amounts of lipids are excreted from fistulas of the ileum than are excreted in the feces on a lipid-free diet would seem to force the conclusion that there is a considerable absorption from the colon. However, it is conceivable that in the normal dog intestinal contents are retained in the small intestine by the action of the ileocecal valve long enough to permit local reabsorption.<sup>3</sup>

In order to test this possibility, cecostomies were performed on three dogs. This operation differed from the one used in making fistulas of the ileum in that the intestine was not sectioned. Instead it was tied off with a double ligature just distal to the cecum, which was straightened out by snipping connective tissue, drawn out through a stab wound, and used for the formation of the fistula.

One of these operations (Dog 251, Table II) was not particularly satisfactory since the fistula tended to close and the dog showed evidence of a partial obstruction, with considerable vomiting. In the remaining two dogs, the fistulas remained in good condition during 3 weeks of experimentation, but in both cases there was considerable passage of material past the ligature into the colon, as evidenced by the excretion of several stools during the experiments and the finding of a small open passage at autopsy.<sup>4</sup> Nevertheless, there was a considerable excretion of material from the fistulas of the cecum which was collected and analyzed as before. It will be observed from Table II, where the results are presented, that, despite the fact that a fairly large portion of the secretions from the small intestine must have been passing into

<sup>3</sup> The authors are indebted to Dr. R. Gordon Sinclair for this suggestion.

<sup>4</sup> All of the stools excreted by Dog 147, except one which was lost, together with the contents of the colon obtained at autopsy, were analyzed by the method used in previous work with feces. The total lipid content was 4.935 gm. and it was found to contain 27.1 per cent of unsaponifiable material and 72.9 per cent of fatty acids.

the colon, there still was an excretion from the fistulas which averaged well above the average excretion by normal dogs, on the basis of dog weight. This finding shows even more definitely than the results with ileum fistulas that there is a relatively large secretion of lipids into the small intestine. Since the ileocecal valve was probably functioning to some extent at least in these experiments, it might be expected that any normal reabsorption process in the small intestine would have had time to occur. These experiments are particularly valuable because it was pos-

TABLE II  
*Lipids from Fistulas of Cecum*

Experi- ment No.	Dog No	Dog weight	Petroleum ether- soluble fraction*	Lipids		Unsaponifiable fraction		
				Un- saponi- fiable + fatty acid fractions	Per kilo dog weight per wk.	Weight	In lipids	Sterols in
		<i>kg</i>	<i>gm</i>	<i>gm.</i>	<i>mg.</i>	<i>gm</i>	<i>per cent</i>	<i>per cent</i>
20	251	5 9	3 614 (None)	2 650	449	0 770	29 0	32 4
21	147	10 5	8 224 (0 006)	5 741	547	1 801	31 4	42 9
22			5 478 (None)	3 870	369	1 568	40.5	32 9
23			4 274 (0 003)	2 997	285	1 220	40 6	57.6
24	203	8 7	5 102 (0.007)	4 110	473	1 372	33.4	32 8
25			3 763 (None)	3 342	384	2 202	65 8	21.4
26			2 236 ( " )	2 020	232	1 167	57 7	23 7
Average . . . . .							42 6	34 8

\* Numbers in parentheses are ether-soluble, petroleum ether-insoluble fractions.

sible to continue them for a long period with no question concerning the condition of the animals.

The foregoing results indicate that there is a large secretion of lipids into the small intestine, but it is possible that the apparent secretion may have originated wholly or in part in (1) bacterial synthesis, (2) digestion of skin, or (3) a secretion of lipids onto the surface of the skin.

*Rôle of Bacteria*—Microscopic examination<sup>5</sup> of the material ex-

<sup>5</sup> The authors are indebted to Dr. Martha Wollstein and Dr. Beryl H. Paige of the Department of Pathology, Babies Hospital, for these examinations.

creted from the fistulas showed large numbers of bacteria. Previous investigations of the rôle of bacteria in lipid excretion were not entirely conclusive. About 40 per cent of the total excretion was found in the portion of the feces which contained most of the bacteria, while most of the remaining 60 per cent was present in other solid particles (3). These results were interpreted to mean that the fecal lipid excretion is contained in bacteria and other cellular elements of the feces; but the possibility was recognized that the excretion might represent in whole or part a secretion of free lipids which had been adsorbed on solid particles of the feces. Later (4) it was found that when fat was fed to bile fistula dogs practically all of the excess fat excretion was adsorbed in the non-bacterial solid elements of the feces.

Von Behring (10) was unable to find a trace of sterols in colon or diphtheria bacilli grown on sterol-free media. This finding was in agreement with the results of Anderson and Chargaff (11), who found no sterols in tubercle bacilli, and Beumer and Hepner (5), who found no sterols in stool cultures. Von Behring states that considerable amounts of sterols were found in the bacterial lipids by one of us (3) and argues that, since bacteria apparently do not contain sterols, it is probable that the lipids found in the bacterial fraction of the feces were adsorbed on and not constituents of the bacteria. Von Behring's argument is a good one; but, as a matter of fact, no sterol determinations were reported in the paper referred to, since at that time it was not known that bacteria are probably sterol-free and that the presence of sterols would indicate a non-bacterial source of the lipids found in the bacterial fraction.

Digitonin-precipitable sterols have now been determined by the method described in the preceding paper in most of the bacterial unsaponifiable fractions obtained in the previous investigations. Altogether 67 fractions have been analyzed, of which thirty-three were from normal and thirty-four from bile fistula dogs. The average sterol (calculated as cholesterol) content of the former was 26.5 and of the latter 47.3 per cent. The finding of considerable amounts of sterols indicates that the lipids present had to a considerable extent at least been adsorbed on, rather than synthesized by bacteria.

In the present investigation the unsaponifiable material obtained from the fistulas contained on the average 42.3 per cent of

sterols (Table I). This value is close to that found in the bacterial fractions from bile fistula dogs and happens to be almost identical with the average of a number of determinations by the colorimetric method of total unsaponifiable fractions from the lipids excreted by normal and bile fistula dogs (2). Similar values were also found in skin (see below), in intestinal mucosa (preceding paper), and in various tissues of *Omnivora* by Lemeland (12), Liang and Wacker (13), Wacker (14), and Channon and Marrian (15). Schönheimer, von Behring, and Hummel (16) found a somewhat higher proportion of sterols in the unsaponifiable material from various human tissues. On the other hand, Igarashi (17) in a very large series of determinations on rabbit tissues found relatively small percentages of cholesterol in unsaponifiable fractions. Apparently rabbits differ from *Omnivora* in this regard, as might be expected.

From the fact that the unsaponifiable material excreted from fistulas contained amounts of sterols similar to the tissues of omnivorous animals, and the failure of several investigators to find sterols in bacteria, it may be concluded that in all probability the lipids excreted did not originate in bacterial synthesis.

*Skin*—A second possible source of the lipids was digestion of skin, which took place to a considerable extent around the fistulas in most of the experiments. To test this possibility portions of skin of approximately the area involved<sup>\*</sup> were removed from the abdomen and sides in a series of normal dogs, the subcutaneous fat was removed as completely as possible, and the total content of unsaponifiable material was determined by the same method used in the study of intestinal mucosa (see preceding paper). The average of twelve such determinations was 52 mg. with a minimum of 37 mg. and a maximum of 90 mg. The average cholesterol content (eight determinations) was 46.7 per cent. It may be computed that on the average the skin in the area involved would have to digest away and be replaced thirty-six times a week to account for the unsaponifiable material found in the excretion. This is far beyond the realm of possibility and digestion of skin cannot introduce a significant error.

<sup>\*</sup> The portions of skin taken averaged about 20 sq. inches in area. The amount was varied with the size of the dog to correspond roughly with the areas in contact with the excretion in dogs of different size.

There still remains the possibility that a *secretion of lipids* through the skin is stimulated by the irritative and digestive action of the intestinal juices in contact with it. Several unsuccessful attempts have been made to avoid contact with the skin by canulizing the fistulas and collecting the excretion in a balloon. Some evidence has been obtained indicating that secretion by the skin is not an important factor. In the cecostomy dogs practically no digestion and irritation of the skin were evident. In the case of Dog 607 the skin about the fistula was coated with rubber cement immediately after the operation. Very little inflammation was apparent. In the case of Dog 307 rubber cement was also used but it peeled off and the skin became rather badly inflamed during the 1st week. During the 2nd week the gauze was saturated with ammonium chloride solution and dried each day before application. The skin improved rapidly and by the end of the week appeared quite normal. Ammonium chloride was also used in the case of Dog 405. The skin showed little inflammation. In all of these cases where the skin was relatively normal the amounts of lipids found were as large as in experiments where the skin showed a good deal of digestion and irritation.

Samples of skin from the area in contact with the excretion and from a nearby normal area were removed at autopsy from Dogs 307 and 405 and examined microscopically.<sup>5</sup> There was no evidence of any accumulation of lipids in the former as compared with the latter.

*Collection of Lipids in Colon*—On the 1st and usually the 2nd day of the lipid-free diet the isolated colon was irrigated with large enemas of dilute Dakin's solution to remove fecal material. In all cases where an autopsy was performed immediately after death the colon was found to contain a material of wax-like nature, not at all fecal in appearance or odor. It was firmly adherent to and, when removed carefully, carried the complete design of the rugæ of the intestinal wall. The portion in immediate contact with the intestinal wall was light yellow in color while in the center it was usually quite red, very much like clotted blood in appearance. In one case where tests were made, very strong guaiacum and hematin crystal reactions were given by this material. Apparently there had been rather copious bleeding from the turned in end of the intestine. No other source of hemorrhage could be seen.

The lipid content of the material from the colon was determined by the total saponification procedure which was used in work with feces. The results are given in Table III and are calculated on a weekly basis. In all but two of the dogs the amount of lipids which collected per kilo per week was smaller than the lowest excretion found in any of the 83 experiments on normal dogs (see p. 759, preceding paper). The average collection was 23 per cent of the average excretion.

While this work was in progress Schönheimer and von Behring (8) reported experiments with dogs which had been subjected to an operation practically identical with the ileostomy employed in this work. They were interested primarily in studying the secretion of sterols into the colon and took great pains to wash it out with several liters of water at operation before closing it with a purse-string suture. The colon was washed daily for a month after the operation with large enemas, after which the anus was closed. At the end of 1 to 2 months the artificial cyst was found to be filled "mit einer glasigen, gelblich-weissen, etwas fade, jedoch niemals faul riechenden elastischen, fast immer sterilen Masse." The description tallies exactly with that of the material found in these experiments except for the absence of blood. Schönheimer and von Behring note the presence of blood in the colon washings for a few days after the operation.

Unfortunately a protocol is given for only one experiment and the dog weight is omitted. In this single experiment 1.05 gm. of chloroform-soluble material collected in the colon during a period of 2 months. Assuming that the dog weighed 10 kilos, this would amount to 11.7 mg. per kilo per week, which is somewhat less than was found in most of the experiments reported in Table III, but only a little below the results of two experiments. However, Schönheimer and von Behring found 80 per cent unsaponifiable material in the lipids while the average in this work was 44.5 per cent. The amount of unsaponifiable material which collected per kilo per week in Schönheimer and von Behring's experiment was, therefore, comparable with the low values found in this work.

Heupke (9) made two colorimetric determinations of the cholesterol excreted from the isolated colon in 24 hours. The average calculated per kilo per week was 4.25 mg. which is considerably less than the average excretion of digitonin-precipitable sterols

found in this work, though in one dog a smaller amount was obtained.

The finding that much larger amounts of lipids are excreted from the small intestine than would be excreted in the feces under the same dietary conditions, while at the same time relatively small amounts of lipids collect in the colon, leads to the unexpected conclusion that in the normal dog there is a considerable absorption of lipids in the colon. The possibility has not been ruled out entirely that the reabsorption in the normal animal takes place in

TABLE III  
*Lipids from Colon*

Dog No.	Dog weight	Petroleum ether-soluble fraction*	Lipids		Unaponifiable fraction		
			Unaponifiable + fatty acid fractions	Per kilo dog weight per wk.	Weight	In lipids	Sterols in
	kg.	gm.	gm.	mg.	gm.	per cent	per cent
215	4.7	0.246 (None)	0.235	50	0.070	29.8	41.4
216	12.3	0.287 (0.009)	0.274	22	0.096	35.0	50.7
218	5.5	0.331 (0.007)	0.312	57	0.131	41.9	49.6
229	11.0	1.105 (0.010)	1.053	96	0.368	34.9	40.2
235	4.7	0.204 (None)	0.183	39	0.067	36.6	50.3
238	14.5	0.266 ( " )	0.240	17	0.093	38.7	45.4
239	4.3	0.076 ( " )	0.070	16	0.031	44.3	13.7
607	20.0	0.877 (0.281)	0.812	41	0.263	32.4	
720	8.3	0.536	0.499	60	0.306	61.4	33.4
307	11.8	1.364	1.242	105	1.119	90.2	54.5
Average.....				50		44.5	42.1

\* Numbers in parentheses are ether-soluble, petroleum ether-insoluble fractions.

the lower portion of the small intestine, but the experiments with cecostomies rather indicate that such is not the case.

Not much accurate information is available in the literature concerning fat absorption from the colon. Czerny and Latschenberger (18) found a rather large absorption of fat emulsions from a blind pouch of the large intestine. Kobert and Koch (19) had a similar patient in whom almost the whole large intestine formed a closed tube. They found that emulsified fat was absorbed slowly in small amounts from this pouch, while non-emulsified fat was

hardly absorbed at all. Wuttig (20) concluded from histological evidence that absorption does take place in the colon. On the other hand, Levites (21), working with intestinal fistulas, found that fat had been mostly absorbed by the time it reached the cecum. Yamakawa, Nomura, and Fujinaga (22) appear to be the only investigators who have studied the question of fat absorption from the colon directly in animals. They present strong evidence for the absorption of emulsions of animal oils and lecithin from the dog colon.

The question arises as to the manner in which lipids are secreted into the small intestine. Schönheimer and von Behring (8) observed in the secretion from the small intestine a large number of lymphocytes which were destroyed rapidly by intestinal ferments. Dr. Schönheimer has suggested (personal communication) that the lipids found in the intestinal excretion are contained to a large extent in these cells. A number of smears of the excretion were examined<sup>5</sup> in the case of Dog 307. Occasional particles were seen to stain brightly with scarlet red, but it was difficult to make out whether they were free droplets of fat or present in cells or fragments of cells. Many bacteria, none of which stained at all, were seen.

In a previous paper (2) it was concluded that bile was not the source of fecal lipids since bile fistula dogs on a lipid-free diet excreted more lipids than normal dogs. It was suggested at that time that the excretion might originate in a secretion into the intestine. In a recent paper Heupke (9) states that this interpretation may not be entirely correct since it is not in agreement with his and Kobert and Koch's finding of a small secretion into the colon, with which finding the results of this investigation are in accord. Because of the small secretion into the colon he includes the bile as a principal source of fecal lipids. He overlooks the fact that the suggestion as made applied to the entire intestinal tract. The results of this investigation show that the excretion originates principally in the small intestine. The finding of a small secretion of lipids into the colon in no way invalidates the conclusion that the bile is not the source of fecal lipids and that the excretion may originate in a secretion into the intestine. It simply shows that the secretion is localized for the most part to the small intestine.



The investigations described in this paper present no evidence either for or against the origin of fecal lipids in the bile. Similar studies on animals with both ileostomies and bile fistulas would settle the matter beyond further doubt; but it is questionable whether dogs would survive such an operation long enough to allow satisfactory studies. Moreover, since it has already been demonstrated conclusively that bile fistula dogs excrete lipids in relatively large amounts, there seems to be no need for such experiments unless it be assumed that the excretion by the bile fistula dog represents a process entirely different from that occurring in dogs with intestinal fistulas.

#### SUMMARY AND CONCLUSIONS

1. Fistulas of the lower ileum have been established in a series of dogs and the lipids excreted therefrom have been determined while the dogs were on a lipid-free diet. Concomitant studies have been made of the lipid secretion into the isolated colon.

2. A much larger excretion of lipids from the small intestine occurred than was found previously in the feces under the same dietary conditions.

3. Evidence is presented indicating that the lipids found do not originate to any appreciable extent in bacterial synthesis or in the skin.

4. In most cases the lipids which collected in the blind colonic stump were less than one-fifth of the amount which was previously found in the feces of similarly fed animals.

5. Similar experiments have been carried out on dogs with fistulas of the cecum and ligatures around the colon in order to retain the action of the ileocecal valve. Large excretions of lipids were found, despite the passage of considerable amounts of small intestinal secretions past the ligatures into the colon.

6. The results indicate that relatively large amounts of lipids are secreted into the small intestine. A considerable portion of this secretion is reabsorbed, apparently in the large intestine, though this is not certain. The remainder, together with a relatively small amount of lipids secreted into the colon, probably makes up the endogenous lipid excretion which has been found previously in feces.

The authors take this opportunity to express their gratitude to Dr. W. R. Bloor for suggesting this problem and for his helpful criticism and advice.

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